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TITLE: Mesenchymal stem cells in the bone marrow provide a supportive niche for early disseminated breast tumor initiating cells

PRINCIPAL INVESTIGATOR: Carolyn G. Marsden, Ph.D.

CONTRACTING ORGANIZATION: Tulane University Health Sciences Center  
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14. ABSTRACT  Tumorspheres were isolated from core biopsies collected from five patients with clinical diagnosis of invasive ductal carcinoma (IDC). Isolated tumorspheres were transplanted into the mammary fat pad of NUDE mice to establish tumorigenicity in vivo. At 3 months post-injection, micrometastases to the lung, liver, kidneys, brain and femur were detected by measuring content of human chromosome 17. Primary tumors variably expressed cytokeratins, Her2/neu, cytoplasmic E-cadherin, nuclear $\beta$ catenin and fibronectin but were negative for ER $\alpha$ and vimentin. The injection of bone marrow isolated from mice previously injected with tumorspheres into the mammary fat pad, resulted in large tumor formation in the mammary fat pad 2 months post-injection. The tumors exhibited accelerated development of metastatic lesions within the lung, liver and kidney. The resultant tumors and the majority of metastatic lesions within the lung and liver exhibited a mesenchymal-like phenotype. Conclusions: Micrometastases in mouse organs demonstrated a dormancy period prior to outgrowth of macrometastases. Dormant disseminated tumor cells (DTCs) within the bone marrow were highly malignant upon injection into the mammary fat pad, with the accelerated development of metastatic lesions. These results indicate the acquisition of a more aggressive phenotype of DTCs during metastatic latency within the bone marrow microenvironment.					
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## INTRODUCTION:

Breast cancer has the highest incidence and second highest mortality in women in the United States. Advances in early detection and treatment modalities have increased the 5 year survival rate for women battling with breast cancer. However, the 10 year survival rate has not increased significantly. The most common metastasis observed in breast cancer patients is bone metastasis. Bone metastasis can remain latent for a long time, being virtually undetectable for years. Upon activation, which involves mechanisms not yet elucidated, metastasized breast cancer cells can begin to proliferate and differentiate into various cancer cell lineages in the bone marrow becoming clinical bone metastasis. The majority of disseminated cancer cells are unable to evade the immune system or undergo apoptosis. Metastasized breast cancer cells in the bone marrow have already been successful in evading the immune system and extravasated from the blood into the bone marrow. Once in the foreign environment of bone marrow, disseminated breast cancer cells require interactions with a supportive niche that promotes their survival and maintains their stem cell phenotype. **I hypothesize that resident mesenchymal stem cells in the bone marrow provide a supportive niche for breast cancer cells that have metastasized to bone marrow.** The success of this type of study lies in applying models that most closely represent the actual physiological environment of breast cancer cells in bone. We developed a model of early disseminated breast cancer in bone from fresh patient biopsies. This study evaluated tumorigenicity, metastatic spread, and the morphological and molecular phenotype of disseminated tumor cells from these tumors.

## BODY:

**Statement of Work.** To fully characterize the phenotype of breast tumor initiating cells in culture, at the primary site of tumor formation and in the bone microenvironment. (Months 1-36)

- a. Perform immunocytochemistry on cytopins of tumorspheres and flow cytometry analysis using antibodies to CD44, CD24, ESA, MUC-1 (CD227), CD10, alpha-smooth muscle actin, cytokeratin 18, cytokeratin 14, estrogen receptor and HER2/neu.
- b. Perform differentiation experiments using breast tumor initiating cells
- c. Characterize the phenotype of breast tumor initiating cells in the primary tumor and in bone using the same antibodies listed above.

The following published manuscripts addressed each aspect of the statement of work above.

**Publication 1.** "Disseminated breast cancer cells acquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone". Carolyn G. Marsden, Mary Jo Wright, Latonya Carrier, Krzysztof Moroz, and Brian G. Rowan (2012). PLoS One. 2012;7(11):e47587. PMID:23173031

## Abstract

**BACKGROUND:** Disseminated tumor cells (DTCs) in the bone marrow may exist in a dormant state for extended periods of time, maintaining the ability to proliferate upon activation, engraft at new sites, and form detectable metastases. However, understanding of the behavior and biology of dormant breast cancer cells in the bone marrow niche remains limited, as well as their potential involvement in tumor recurrence and metastasis. Therefore, the purpose of this study was to investigate the tumorigenicity and metastatic potential of dormant disseminated breast cancer cells (prior to activation) in the bone marrow.

**METHODOLOGY/PRINCIPAL FINDINGS:** Total bone marrow, isolated from mice previously injected with tumorspheres into the mammary fat pad, was injected into the mammary fat pad of NUDE mice. As a negative control, bone marrow isolated from non-injected mice was injected into the mammary fat pad of NUDE mice. The resultant tumors were analyzed by immunohistochemistry for expression of epithelial and mesenchymal markers. Mouse lungs, livers, and kidneys were analyzed by H+E staining to detect metastases. The injection of bone marrow isolated from mice previously injected with tumorspheres into the mammary fat pad, resulted in large tumor formation in the mammary fat pad 2 months post-injection. However, the injection of bone marrow isolated from non-injected mice did not result in tumor formation in the mammary fat pad. The DTC-derived

tumors exhibited accelerated development of metastatic lesions within the lung, liver and kidney. The resultant tumors and the majority of metastatic lesions within the lung and liver exhibited a mesenchymal-like phenotype.

**CONCLUSIONS/SIGNIFICANCE:** Dormant DTCs within the bone marrow are highly malignant upon injection into the mammary fat pad, with the accelerated development of metastatic lesions within the lung, liver and kidney. These results suggest the acquisition of a more aggressive phenotype of DTCs during metastatic latency within the bone marrow microenvironment.

**Publication 2.** "A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies". Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. BMC Cancer. 2012 Jan 10;12:10. PMID: 22233382

#### Abstract

**BACKGROUND:** The study of breast cancer metastasis depends on the use of established breast cancer cell lines that do not accurately represent the heterogeneity and complexity of human breast tumors. A tumor model was developed using primary breast tumor-initiating cells isolated from patient core biopsies that would more accurately reflect human breast cancer metastasis.

**METHODS:** Tumorspheres were isolated under serum-free culture conditions from core biopsies collected from five patients with clinical diagnosis of invasive ductal carcinoma (IDC). Isolated tumorspheres were transplanted into the mammary fat pad of NUDE mice to establish tumorigenicity in vivo. Tumors and metastatic lesions were analyzed by hematoxylin and eosin (H+E) staining and immunohistochemistry (IHC).

**RESULTS:** Tumorspheres were successfully isolated from all patient core biopsies, independent of the estrogen receptor  $\alpha$  (ER $\alpha$ )/progesterone receptor (PR)/Her2/neu status or tumor grade. Each tumorsphere was estimated to contain 50-100 cells. Transplantation of 50 tumorspheres ( $1-5 \times 10^3$  cells) in combination with Matrigel into the mammary fat pad of NUDE mice resulted in small, palpable tumors that were sustained up to 12 months post-injection. Tumors were serially transplanted three times by re-isolation of tumorspheres from the tumors and injection into the mammary fat pad of NUDE mice. At 3 months post-injection, micrometastases to the lung, liver, kidneys, brain and femur were detected by measuring content of human chromosome 17. Visible macrometastases were detected in the lung, liver and kidneys by 6 months post-injection. Primary tumors variably expressed cytokeratins, Her2/neu, cytoplasmic E-cadherin, nuclear  $\beta$  catenin and fibronectin but were negative for ER $\alpha$  and vimentin. In lung and liver metastases, variable redistribution of E-cadherin and  $\beta$  catenin to the membrane of tumor cells was observed. ER $\alpha$  was re-expressed in lung metastatic cells in two of five samples.

**CONCLUSIONS:** Tumorspheres isolated under defined culture conditions from patient core biopsies were tumorigenic when transplanted into the mammary fat pad of NUDE mice, and metastasized to multiple mouse organs. Micrometastases in mouse organs demonstrated a dormancy period prior to outgrowth of macrometastases. The development of macrometastases with organ-specific phenotypic distinctions provides a superior model for the investigation of organ-specific effects on metastatic cancer cell survival and growth.

**Publication 3.** Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. Marsden CG, Wright MJ, Pochampally R, Rowan BG. Methods Mol Biol. 2009;590:363-75. doi: 10.1007/978-1-60327-378-7\_23. PMID:19763516

#### Abstract:

In recent years, evidence has emerged supporting the hypothesis that cancer is a stem cell disease. The cancer stem cell field was led by the discovery of leukemia stem cells (Tan, B.T., Park, C.Y., Ailles, L.E., and Weissman, I.L. (2006) The cancer stem cell hypothesis: a work in progress. Laboratory Investigation. 86, 1203-1207), and within the past few years cancer stem cells have been isolated from a number of solid tumor including those of breast and brain cancer among others (Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. USA 100, 3983-3988; Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J., and Dirks, P.B. (2003) Identification of a Cancer Stem Cell in Human Brain Tumors. Cancer Research. 63, 5821-5828). Cancer stem cells exhibit far different properties than established cells lines such as relative quiescence, multidrug resistance, and multipotency (Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J.,

Jamieson, C.H.M., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006) Cancer Stem Cells- Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Research*. 66, 9339-9344). In addition, our laboratory has demonstrated that breast cancer stem cells exhibit a strong metastatic phenotype when passaged in mice. Since stem cells exhibit these somewhat unique properties, it will be important for endocrinologists to evaluate hormonal action in these precursor cells for a more thorough understanding of cancer biology and development of more effective treatment modalities. A relatively easy and low cost method was developed to isolate breast cancer stem cells from primary needle biopsies taken from patients diagnosed with primary invasive ductal carcinoma during the routine care of patients with consent and IRB approval. Fresh needle biopsies (2-3 biopsies at 2 cm in length) were enzymatically dissociated in a collagenase (300 U/ml)/hyaluronidase (100 U/ml) solution followed by sequential filtration. Single cell suspensions were cultured on ultra low attachment plastic flasks in defined medium and formed non-adherent tumorspheres. The tumorspheres exhibited surface marker expression of CD44(+)/CD24(low-)/ESA(+), previously defined as a "breast cancer stem cell" phenotype by Al Hajj et al. (Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells.

## **KEY RESEARCH ACCOMPLISHMENTS:**

- Tumorspheres were successfully isolated from all patient core biopsies. Transplantation of 50 tumorspheres into the mammary fat pad of NUDE mice resulted in small, palpable tumors that were sustained up to 12 months post-injection.
- Primary tumors variably expressed cytokeratins, Her2/neu, cytoplasmic E-cadherin, nuclear  $\beta$  catenin and fibronectin but were negative for ER $\alpha$  and vimentin.
- At 3 months post-injection, micrometastases to the lung, liver, kidneys, brain and femur were detected
- Micrometastases in mouse organs demonstrated a dormancy period prior to outgrowth of macrometastases.
- The injection of bone marrow isolated from mice previously injected with tumorspheres into the mammary fat pad, resulted in large tumor formation in the mammary fat pad 2 months post-injection.
- The DTC-derived tumors exhibited accelerated development of metastatic lesions within the lung, liver and kidney.
- The resultant tumors and the majority of metastatic lesions within the lung and liver exhibited a mesenchymal-like phenotype.
- The development of macrometastases with organ-specific phenotypic distinctions provides a superior model for the investigation of organ-specific effects on metastatic cancer cell survival and growth.

## **REPORTABLE OUTCOMES:**

### **1. Manuscripts, abstracts, presentations;**

#### **Manuscripts:**

**Publication 1.** "Disseminated breast cancer cells acquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone". Carolyn G. Marsden, Mary Jo Wright, Latonya Carrier, Krzysztof Moroz, and Brian G. Rowan (2012). *PLoS One*. 2012;7(11):e47587. PMID:23173031

**Publication 2.** "A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies". Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. BMC Cancer. 2012 Jan 10;12:10. PMID: 22233382

**Publication 3.** Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. Marsden CG, Wright MJ, Pochampally R, Rowan BG. Methods Mol Biol. 2009;590:363-75. doi: 10.1007/978-1-60327-378-7\_23. PMID:19763516

### **Abstracts:**

**Marsden C**, Wright MJ, Carrier L, Krzysztof K, Pochampally R, Rowan B. 2011. "Primary Breast Cancer Cells With Metastatic Potential Isolated From Human Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2010. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" CTRC-AACR Annual San Antonio Breast Cancer Symposium.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" e.Hormone.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Louisiana Cancer Research Consortium Annual Retreat.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

**Marsden C**, Wright MJ, Thiyagarajah R, Li Z, Pochampally R, Rowan B. 2008. The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis-"Isolation of tumor initiating cells with metastatic potential from human primary invasive ductal carcinoma" AACR Special Conference: The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis, Los Angeles, CA.

**Marsden C**, Wright MJ, Pochampally R, Rowan B. 2007. "Isolation and characterization of breast cancer stem cells from primary tumor biopsies" Biomedical Sciences Program Scientific Retreat, Tulane University Health Sciences Center.

### **Presentations:**

2011 Tulane Health Sciences Research Days Award for Excellence in Research and Presentation by a Graduate Student, *"Primary Breast Cancer Cells with Metastatic Potential Isolated from Human Invasive Ductal Carcinoma"*.

2009 Invited Poster/Discussion presentation for the 2009 San Antonio Breast Cancer Conference *"Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma"*\*

2008      Invited for oral presentation at the AACR Special conference “*The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis*” 2008, Los Angeles, CA\*

2008      AACR-Aflac, Inc. Scholar-in-Training Award, AACR Special conference “*The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis*” 2008, Los Angeles, CA\*

## **2. Degrees obtained that are supported by this award**

- Carolyn Marsden was awarded a Ph.D. in Biomedical Sciences from Tulane University School of Medicine, 2012

## **3. Employment or research opportunities applied for and/or received based on experience/training supported by this award**

- Carolyn Marsden began a postdoctoral fellowship position with Joann Sweasy M.D and Susan Wallace, Ph.D. at the University of Vermont in 2013.

## **CONCLUSION:**

Tumorspheres isolated from patient core biopsies were tumorigenic when transplanted into the mammary fat pad of NUDE mice, and metastasized to multiple mouse organs. Micrometastases in mouse organs demonstrated a dormancy period prior to outgrowth of macrometastases. Dormant tumor cells within the bone marrow were highly malignant upon injection into the mammary fat pad, with the accelerated development of metastatic lesions within the lung, liver and kidney. These results indicate the acquisition of a more aggressive phenotype of DTCs during metastatic latency within the bone marrow microenvironment. In addition this study has developed a superior model for the investigation of organ-specific effects on metastatic cancer cell survival and growth.

## **REFERENCES:**

"A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies". Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. BMC Cancer. 2012 Jan 10;12:10. PMID: 22233382

"Disseminated breast cancer cells acquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone". Carolyn G. Marsden, Mary Jo Wright, Latonya Carrier, Krzysztof Moroz, and Brian G. Rowan (2012). PLoS One. 2012;7(11):e47587. PMID:23173031

Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. Marsden CG, Wright MJ, Pochampally R, Rowan BG. Methods Mol Biol. 2009;590:363-75. doi: 10.1007/978-1-60327-378-7\_23. PMID:19763516

## **APPENDICES:**

1. CV Carolyn Marsden, Ph.D.
2. Publication 1



"A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies". Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. BMC Cancer. 2012 Jan 10;12:10. PMID: 22233382

3. Publication 2

"Disseminated breast cancer cells acquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone". Carolyn G. Marsden, Mary Jo Wright, Latonya Carrier, Krzysztof Moroz, and Brian G. Rowan (2012). PLoS One. 2012;7(11):e47587. PMID:23173031

4. Publication 3

Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. Marsden CG, Wright MJ, Pochampally R, Rowan BG. Methods Mol Biol. 2009;590:363-75. doi: 10.1007/978-1-60327-378-7\_23. PMID:19763516

**Carolyn G. Marsden**  
1420 Adams Street  
New Orleans, LA 70118  
504-988-1562  
e-mail: cmarsden@tulane.edu

**Education:**

**Tulane University**

**Ph.D., Biomedical Sciences Graduate Program 2012**

Advisor: Brian G. Rowan

GPA: 3.94/4.0

New Orleans, LA 70112

Dissertation: *"A New Model for the Study of Human Breast Cancer Metastasis"*

**State University of New York at Stony Brook**

**B.S., *summa cum laude***

**May 2002**

Major: Biology-Neuroscience tract

Major: Psychology

Cumulative GPA: 3.85/4.0

Stony Brook, NY

**Saint Michaels College**

**September 1998-May 1999**

Major: Biology

GPA: 3.8/4.0

Colchester, VT

**Professional experience:**

**June 2007-Present Tulane University Health Sciences Center**

Biomedical Sciences Graduate Program

Laboratory of Dr. Brian G. Rowan

Department of Structural and Cellular Biology

Responsibilities:

- Effectively plan and execute experiments
- Generate and interpret data
- Present data at seminars and conferences
- Participate in journal club
- Participate in stem cell group meetings
- Prepare manuscripts, abstracts and posters for publication

**October 2005-August 2006 Mount Sinai Medical Center**

Laboratory of Dr. Margaret H. Baron

Department of Hematology/Oncology

Research Coordinator

Responsibilities:

- Maintain hematopoietic stem cell lines
- Create new stem cell lines and analyze
- Perform differentiation experiments

- Preparation of hematopoietic stem cells for FACS

**June 2005-September 2005    State University of New York at Stony Brook**

Laboratory of Dr. Dan Dykhuizen  
Department of Ecology and Evolution  
Temporary Laboratory Technician

**Responsibilities:**

- Preparation of plasmids
- Sequencing various bacterial genes
- Providing support to graduate students and high school interns for various projects ongoing

**May 2003-May 2005    Cold Spring Harbor Laboratories**

Laboratory of Dr. Gregory Hannon  
Molecular Biology Laboratory  
Laboratory Technician

**Responsibilities:**

- Maintenance and genotyping multiple mouse colonies
- Designing and executing new genotyping strategies for transgenic mouse colony
- Preparation of mouse embryonic fibroblasts from mouse embryos
- Preparation of competent bacteria for common use
- Preparation of other common reagents
- Maintenance of a tissue culture facility shared between multiple laboratories
- General organization and maintenance of a laboratory, consisting of approximately 17 members, for proper functioning

**2002- 2003        Boston Medical Center**

Laboratory of Dr. Thomas Rothstein  
Department of Immunology  
Research Technician

**Responsibilities:**

- Dialyzing and testing supernatants
- Making buffers and solutions
- Maintaining records on mouse colonies
- Ordering supplies and equipment for the laboratory
- Autoclaving materials/glassware
- Performing monthly radiation inspections and maintaining organized reports

**1998-1999        Hofstra University**

Laboratory of Dr. Robert Seagull  
Biology Department  
Cellular Biology Laboratory

Summer internship

**Responsibilities:**

- Making agar plates
- Maintaining cotton plant population
- Glassware

**Research Laboratory Skills:**Mouse Embryology:

- Micro-dissections of mouse embryos (day 7.5-day 16.5)
- Preparation and maintenance of MEFs from mouse embryos
- Beta-Gal staining of Gene Trap embryos
- In situ hybridization
- Designing genotyping strategies for mouse colonies

Molecular Biology:

- PCR
- Southern Blotting
- RNA extraction/gels
- Molecular Cloning
- DNA preparation
- Electrophoresis

Cell Culture:

- Freezing/thawing cell lines
- Transfection of various cell types
- Drug selection of cell populations
- Culturing mouse embryonic stem cells
- Fluorescent microscopy
- Apoptosis assays
- Electroporation of mouse embryonic stem cells
- Differentiation of mouse embryonic stem cells

Immunology:

- Flow cytometry
- Proliferation Assays
- Chromium Release Assays
- B cell preparation from the extracted spleens of mice
- B cell preparation from peritoneal washes

Trained to work with various radioisotopes including:  $P^{32}$ ,  $Cr^{51}$  and  $H^3$

**Teaching Experience:***Tulane University*

- Teaching Assistant, Department of Cell and Molecular Biology
- Taught undergraduate non-science majors in Heredity and Society Lab

**Research Interests:**

- Cancer Biology/Genetics
- Cancer metastasis
- Stem cell biology
- Tissue morphogenesis
- Extracellular matrix biology

- Development and improvement of research techniques

### Publications:

Disseminated breast cancer cells acquire a highly malignant and aggressive metastatic phenotype during metastatic latency in the bone. Marsden CG, Wright MJ, Carrier L, Moroz K, Rowan BG. PLoS One. 2012;7(11):e47587. doi: 10.1371/journal.pone.0047587. Epub 2012 Nov 15. PMID:23173031

Peptidomimetic Src/pretubulin inhibitor KX-01 alone and in combination with paclitaxel suppresses growth, metastasis in human ER/PR/HER2-negative tumor xenografts. Anbalagan M, Ali A, Jones RK, Marsden CG, Sheng M, Carrier L, Bu Y, Hangauer D, Rowan BG. Mol Cancer Ther. 2012 Sep;11(9):1936-47. doi: 10.1158/1535-7163.MCT-12-0146. Epub 2012 Jul 10. PMID: 22784709

Stable inhibition of specific estrogen receptor  $\alpha$  (ER $\alpha$ ) phosphorylation confers increased growth, migration/invasion, and disruption of estradiol signaling in MCF-7 breast cancer cells. Huderson BP, Duplessis TT, Williams CC, Seger HC, Marsden CG, Pouey KJ, Hill SM, Rowan BG. Endocrinology. 2012 Sep;153(9):4144-59. doi: 10.1210/en.2011-2001. Epub 2012 Jun 25. PMID:22733972

"A novel in vivo model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies". Marsden CG, Wright MJ, Carrier L, Moroz K, Pochampally R, Rowan BG. BMC Cancer. 2012 Jan 10;12:10. doi: 10.1186/1471-2407-12-10. PMID:22233382

Breast tumor-initiating cells isolated from patient core biopsies for study of hormone action. Marsden CG, Wright MJ, Pochampally R, Rowan BG. Methods Mol Biol. 2009;590:363-75. doi: 10.1007/978-1-60327-378-7\_23. PMID:19763516

Argonaute 2 is the Catalytic Engine of Mammalian RNAi. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song J, Hammond SM, Joshua-Tor L, Hannon GJ. Science. 2004 Sept 3; 305: 1437-1441.

### Abstracts:

**Marsden C**, Wright MJ, Carrier L, Krzysztof K, Pochampally R, Rowan B. 2011. "Primary Breast Cancer Cells With Metastatic Potential Isolated From Human Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2010. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" CTRC-AACR Annual San Antonio Breast Cancer Symposium.

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**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Louisiana Cancer Research Consortium Annual Retreat.

**Marsden C**, Wright MJ, Carrier L, Pochampally R, Rowan B. 2009. "Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma" Tulane Health Sciences Research Days.

**Marsden C**, Wright MJ, Thiyagarajah R, Li Z, Pochampally R, Rowan B. 2008. The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis-"Isolation of tumor initiating cells with metastatic potential from human primary invasive ductal carcinoma" AACR Special Conference: The Role of Cancer Stem Cells in the

Initiation and Propagation of Tumorigenesis, Los Angeles, CA.

**Marsden C**, Wright MJ, Pochampally R, Rowan B. 2007. "Isolation and characterization of breast cancer stem cells from primary tumor biopsies" Biomedical Sciences Program Scientific Retreat, Tulane University Health Sciences Center.

**Grants:**

2010-2012 DOD Individual Predoctoral Fellowship, "*Mesenchymal stem cells in the bone marrow provide a supportive niche for early disseminated breast tumor initiating cells.*"

2006-2010 Louisiana Board of Regents Individual Predoctoral Fellowship, 2006-2010

**Awards/Honors:**

2011 Tulane Health Sciences Research Days Award for Excellence in Research and Presentation by a Graduate Student, "*Primary Breast Cancer Cells with Metastatic Potential Isolated from Human Invasive Ductal Carcinoma*".

2009 Invited Poster/Discussion presentation for the 2009 San Antonio Breast Cancer Conference "*Isolation of Tumor Initiating Cells with Metastatic Potential from Human Primary Invasive Ductal Carcinoma*"\*

2008 Invited for oral presentation at the AACR Special conference "*The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis*" 2008, Los Angeles, CA\*

2008 AACR-Aflac, Inc. Scholar-in-Training Award, AACR Special conference "*The Role of Cancer Stem Cells in the Initiation and Propagation of Tumorigenesis*" 2008, Los Angeles, CA\*

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RESEARCH ARTICLE

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# "A novel *in vivo* model for the study of human breast cancer metastasis using primary breast tumor-initiating cells from patient biopsies"

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## Abstract

**Background:** The study of breast cancer metastasis depends on the use of established breast cancer cell lines that do not accurately represent the heterogeneity and complexity of human breast tumors. A tumor model was developed using primary breast tumor-initiating cells isolated from patient core biopsies that would more accurately reflect human breast cancer metastasis.

**Methods:** Tumorspheres were isolated under serum-free culture conditions from core biopsies collected from five patients with clinical diagnosis of invasive ductal carcinoma (IDC). Isolated tumorspheres were transplanted into the mammary fat pad of NUDE mice to establish tumorigenicity *in vivo*. Tumors and metastatic lesions were analyzed by hematoxylin and eosin (H+E) staining and immunohistochemistry (IHC).

**Results:** Tumorspheres were successfully isolated from all patient core biopsies, independent of the estrogen receptor  $\alpha$  (ER $\alpha$ )/progesterone receptor (PR)/Her2/neu status or tumor grade. Each tumorsphere was estimated to contain 50-100 cells. Transplantation of 50 tumorspheres ( $1-5 \times 10^3$  cells) in combination with Matrigel into the mammary fat pad of NUDE mice resulted in small, palpable tumors that were sustained up to 12 months post-injection. Tumors were serially transplanted three times by re-isolation of tumorspheres from the tumors and injection into the mammary fat pad of NUDE mice. At 3 months post-injection, micrometastases to the lung, liver, kidneys, brain and femur were detected by measuring content of human chromosome 17. Visible macrometastases were detected in the lung, liver and kidneys by 6 months post-injection. Primary tumors variably expressed cytokeratins, Her2/neu, cytoplasmic E-cadherin, nuclear  $\beta$  catenin and fibronectin but were negative for ER $\alpha$  and vimentin. In lung and liver metastases, variable redistribution of E-cadherin and  $\beta$  catenin to the membrane of tumor cells was observed. ER $\alpha$  was re-expressed in lung metastatic cells in two of five samples.

**Conclusions:** Tumorspheres isolated under defined culture conditions from patient core biopsies were tumorigenic when transplanted into the mammary fat pad of NUDE mice, and metastasized to multiple mouse organs. Micrometastases in mouse organs demonstrated a dormancy period prior to outgrowth of macrometastases. The development of macrometastases with organ-specific phenotypic distinctions provides a superior model for the investigation of organ-specific effects on metastatic cancer cell survival and growth.

**Keywords:** Primary breast tumor-initiating cells, Metastasis, Dormancy, EMT

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## Background

Breast cancer is a heterogeneous disease that remains the second leading cause of death among women. Metastatic disease increases mortality from breast cancer by 70% and is the leading cause of death in breast cancer patients independent of the manageability of the primary disease. Although generally correlated with later stages in disease progression, there is mounting evidence suggesting the metastatic process may initiate earlier in breast cancer development. Therefore, tumor volume at diagnosis may not accurately predict the presence of metastatic disease or the initiation of the metastatic process. Metastatic disease can remain dormant and undetectable for months to years, resulting in recurrence at the primary site and/or the development of metastatic lesions at distant sites [1,2]. Efficacious treatments for metastatic disease depends on development of preclinical tumor models that better predict patient response, increase understanding of the metastatic process, and enable the identification of biomarkers for earlier and more accurate detection of metastasis.

The study of breast cancer has depended heavily upon the use of established breast cancer cell lines, whose origin is often from pleural effusions or metastatic lesions. Although significant advancements have been made possible through the use of established cell lines, further progress depends on the development of tumor models that more accurately represent the heterogeneous nature of human breast tumors. Hetero-transplantation of primary tumor biopsies from patients into immune-deficient mice has many advantages over standard xenografts from cancer cell lines. The hetero-transplant tumors can be directly compared to the original patient tumor biopsies, and to annotated information on patient features, family history, patient outcome etc. A study of breast cancer hetero-transplants revealed that patients whose breast cancer biopsies grew as tumors in mice predicted a worse prognosis compared to biopsies that did not grow tumors [3]. Unfortunately, only a very small percentage of human breast tumor tissue directly transplanted into immune-deficient mice results in tumor formation [3-5]. The identification of breast cancer stem cells (bCSCs) in breast tumors shifted the previously held hypothesis that all cells within a tumor retained the ability to recapitulate the tumor [6]. bCSCs, present in tumors at very low frequency [7], have been implicated in breast tumor progression [8], metastasis [9] and recurrence [10]. The relative quiescence of bCSCs [11] and the elevated expression of ABC transporter family of proteins [12] may contribute to bCSCs evasion of traditional chemotherapy and radiotherapy. Furthermore, recent data has shown that chemotherapeutics [13,14] and radiation [15] may enrich for bCSCs, possibly increasing risk of recurrence.

A subset of cells isolated from primary breast tumors are termed breast tumor-initiating cells (bTICs) for the ability to form tumors upon injection of low numbers into the mammary fat pad of immune-deficient mice [7]. bTICs consist of a heterogeneous population of cells that include a small percentage of bCSCs as well as a range of less to more differentiated progenitor cells. bTICs have been shown to exist *in vitro* as tumorspheres upon selection under non-adherent, serum-free conditions [16]. Recently, it has been suggested that bTICs are the cells within tumors with metastatic potential and the ability to “seed” in distant organs [17]. Therefore, the challenges in targeting bTICs likely extend from the primary site of tumor formation to distant metastatic sites as well. Given the evidence that supports bTICs as the cells with metastatic potential and the source of breast cancer recurrence, tumor models that employ bTICs isolated directly from patient biopsies may provide a more reliable means for study of the metastatic process and tumor recurrence.

Disseminated breast cancer cells may be present at distant sites at the time of primary diagnosis of breast cancer in patients that exhibit no outward signs of clinical metastasis [18,19]. Although current models of breast cancer metastasis have provided great insight into some of the contributing molecular mechanisms, these models have failed to recapitulate the dormancy period observed clinically. Exit from the dormant state is necessary for the development of macro-metastatic lesions in distant organs, yet the mechanisms involved are poorly understood [17,20,21]. The purpose of this study was to develop a novel and reproducible breast cancer model using bTICs isolated as tumorspheres from patient biopsies for the investigation of the metastatic process.

## Methods

### Isolation of tumorspheres

Tumorspheres were isolated using a procedure previously described by this laboratory [22] and derived from Dontu et al. [23]. Briefly, breast cancer needle biopsies from primary patient tumors were obtained during the routine care of patients with consent and Tulane IRB approved protocol (IRB # 07-00042). Biopsies were performed using a 14 gauge spring-loaded gun yielding about 15-20 mg of tissue in each core sample. 3-5 core biopsy samples (2 cm in length) were obtained from each consenting patient. Tissue samples were placed on ice in 1× Hanks buffered saline solution (HBSS) until processing. Tissues were mechanically dissociated using sterile scalpels into ~2 mm<sup>2</sup> pieces followed by enzymatic dissociation in collagenase (300 U/ml) and hyaluronidase (100 U/ml) (Stem Cell Technologies) diluted in complete DMEM/F12 media (see below)

for 3-5 h at 37 deg with agitation every 20-25 min. The resultant cell suspension was sequentially filtered through a 100  $\mu$ m and 40  $\mu$ m pore filter (Fisher) and centrifuged at  $300 \times g$  for 10 min. The cell pellet was resuspended in complete DMEM/F12 media (see below) and cultured in a 100 mm<sup>2</sup> ultra low attachment plate (Corning).

#### Cell culture

Cells isolated from tissue samples were incubated in DMEM/F12 media containing  $1 \times$  B-27 serum-free supplement (Invitrogen), 0.4% bovine serum albumin (BSA) (Sigma), 20 ng/ml epidermal growth factor (EGF) (Sigma), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma), 4  $\mu$ g/ml insulin, human recombinant (Sigma), and Penicillin (100 U/ml)/Streptomycin (100 U/ml). Cells were cultured for 10-14 days to allow tumorsphere formation. Cells were pelleted every 3 days by centrifugation at  $300 \times g$  for 10 min. and resuspended in complete DMEM/F12 media supplemented with fresh EGF and bFGF.

#### Animal experiments

Immunodeficient Nu/Nu female mice were purchased from Charles River Laboratories (US). Mice were 25-35 days of age at time of tumorsphere injection. All experiments were performed under approved Tulane IACUC protocol (IACUC # 2941 R-D). Tumorspheres were washed twice with cold  $1 \times$  PBS then resuspended to yield 1000-5000 cells/100  $\mu$ l in cold  $1 \times$  PBS. Immediately before injection, cells were combined with 100  $\mu$ l BD Matrigel Basement Membrane matrix (BD Biosciences). Mice were anesthetized by i.p. injection of 0.3 ml of a ketamine solution. Cell suspensions were injected bilaterally into the third mammary fat pad. Mice were monitored weekly for tumor formation by caliper measurement, and for body weight for up to 12 months. If no weight loss or other indications of declining health was observed, animals were euthanized 12 months post injection to permit detection of metastases, and to harvest fresh tumor for serial transplantation.

#### DNA isolation and PCR analysis of tumors and mouse tissues

For DNA analysis, tissues were collected using autoclaved dissection tools and placed immediately into sterile polypropylene 15 ml conical tubes at a ratio of 5 ml RNeasy lysis buffer (Qiagen) per 200 mg of tissue. Tissues were either processed immediately or stored at -20 deg. Tissues were homogenized using an electric homogenizer (TH-01, Omni TH, tissue homogenizer) at 25,000 rpm for 2 min. with autoclaved Omni homogenizer tips (8 mm diameter, 110 mm length, processing range of 0.25-30 ml) (Omni International). Cell lysis and all

subsequent steps for the isolation of DNA and RNA from the homogenized tissues were carried out as described in the instruction manual for the Allprep DNA/RNA isolation kit (Qiagen). Briefly, homogenized tissues were loaded onto a spin column that bound DNA and eluted the fraction containing RNA following centrifugation at room temperature. The eluent was combined with 70% ethanol and added to a 2nd spin column. Following several washes, RNA was eluted and quantified. DNA was also eluted from the 1st spin column and quantified. Human cells were detected in mouse tissues using PCR for detection of an alpha-satellite DNA sequence of the centromere region of human chromosome 17 as previously described by Becker et al [24].

#### Hematoxylin and eosin staining

Tissues were collected and placed in 10% neutral buffered formalin (Fisher) equal to 20 times the tissue volume. Tissues were incubated overnight at room temperature and then processed by standard formalin fixation, paraffin embedding and sectioning by The Center for Gene Therapy Histology Core Facility, Tulane University. 5  $\mu$ m sections were deparaffinized and rehydrated in a graded series of ethanol solutions, from 100% to 75%. Sections were then stained using Gill's Hematoxylin and Eosin (Poly Scientific) followed by dehydration through a graded series of ethanol solutions from 75% to 100%. Image J software was used to quantify the metastatic burden within the tissues analyzed. To calculate the metastatic burden present in the mouse organs, the number of pixels within the defined area of the metastatic lesion/s was determined (x pixels). Next, the total number of pixels within the field of view was determined (y pixels). The metastatic burden within the field of view was then calculated by dividing the pixels present in the metastatic lesion/s, by the total pixels comprising the field of view then multiplying by 100 [(x pixels/y pixels)\*100] resulting in a percent metastatic burden. The average of 5 fields of view (100 $\times$  magnification) was used to determine metastatic burden present in each organ analyzed.

#### TUNEL (TdT-mediated dUTP nick end labeling)

For TUNEL, 5  $\mu$ m sections were rehydrated (as described above) followed by heat-induced, epitope retrieval performed in a pressure cooker for 45 min in pH 6.0 Citrate buffer (Biocare Medical). Sections were allowed to cool for 20 min at room temperature. Sections were immersed for 30 min at room temperature in blocking solution in 0.1 M Tris-HCl (pH 7.5) containing 3% BSA and 20% fetal bovine serum (FBS). Sections were rinsed twice with PBS at room temperature. Positive control sections were incubated with 0.5  $\mu$ g/ml

DNase I diluted in a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl<sub>2</sub> and 1 mg/ml BSA at room temperature for 10 min. The sections were incubated in the TUNEL reaction mixture (as supplied by *In Situ* Cell Death Detection kit, Roche) for 60 min at 37°C in a humidified chamber. Sections were washed three times for 5 min in PBS, and then incubated for 10 min at room temperature in 0.3% H<sub>2</sub>O<sub>2</sub> diluted in methanol. To block nonspecific binding of the anti-fluorescein antibody, the sections were incubated in blocking solution (as described above) for 30 min at room temperature then rinsed in PBS. Sections were incubated in Converter-POD (as supplied by *In Situ* Cell Death Detection kit, Roche), diluted in blocking solution (as described above), for 30 min at 37°C in a humidified chamber. Sections were rinsed three times for 5 min each in PBS. The signal was developed using the Vector DAB substrate kit, according to the manufacturers' instructions. Sections were dehydrated (as described above) and mounted using Permount (Fisher).

#### Immunohistochemistry of tumors and mouse tissue

For immunohistochemistry (IHC), tissues were collected and placed in 10% neutral buffered formalin equal to 20 times the tissue volume (Fisher). Tissues were incubated overnight at room temperature and then processed by standard formalin fixation, paraffin embedding and sectioning by The Center for Gene Therapy Histology Core Facility, Tulane University. Immunohistochemistry was performed as per the Vectastain staining kit (anti-rabbit, PK6101; anti-mouse PK6102, Vector Laboratories). Briefly, 5 µm sections were rehydrated (as described above) followed by heat-induced, epitope retrieval performed in a pressure cooker for 25 min. in Tris Buffer, pH 9 (Biocare Medical) or 45 min in pH 6.0 Citrate buffer (Biocare Medical). To inactivate the endogenous peroxide, slides were incubated in 0.3% hydrogen peroxide followed by a 10 min wash in dH<sub>2</sub>O then washed 3 × 3 min. each in PBS. Sections were incubated in blocking buffer (10% normal goat serum diluted in PBS) for 30 min. at room temp and subsequently incubated with primary antibody diluted in blocking buffer overnight at 4 deg. Primary monoclonal antibodies used were rabbit anti-human Ki-67 (SP6, Thermo Scientific), rabbit anti-human E-cadherin (24E10, Cell Signaling), rabbit anti-human Vimentin (SP20, Vector Laboratories), rabbit anti-human Estrogen receptor (SP1, Thermo Scientific), and anti-human HNA (MAB1281, Chemicon). Primary polyclonal antibodies used were rabbit anti-human β-catenin (9563, Cell Signaling), rabbit anti-human fibronectin (ab2413, Abcam), and rabbit anti-human (human specific) cytokeratin 8 (ab52949, Abcam). The following day, sections were washed 2 × 5 min. in PBS-T. Biotinylated secondary antibody was added to the sections for

an incubation period of 30 min, followed by 2 × 5 min. washes in PBS-T. Streptavidin/biotin HRP-conjugate was added to the sections for an incubation period of 30 min. at room temperature followed by 2 × 5 min. washes in PBS-T. The signal was developed using the Vector DAB substrate kit, according to the manufacturers' instructions. Sections were dehydrated (as described above) and mounted using Permount (Fisher). Staining was visualized using a bright field microscope and IP lab software. For quantitation, five randomly selected bright field microscope images (magnification 200×) per sample were obtained as described above. The total cell number in each image was calculated by counting hematoxylin-positive cells using Image J particle count command, and DAB-positive cells were also counted the same way after performing color deconvolution command and expressed as % positive cells.

#### Histological scoring

Her 2 expression within the tumors was measured by IHC and assessed using the histoscore method developed by Allred *et. al.* [25]. Briefly, a proportion score and an intensity score were determined for each tumor sample. The proportion score represented the percentage of positively stained cells (0 = none; 1 = < 5%; 2 = 5-25%; 3 = 26-50% 4 = 51-75% 5 = > 75%) [26]. The intensity score represented the staining intensity in positively stained cells (0 = none; 1 = + weak; 2 = ++ intermediate; 3 = +++ strong). The overall expression of Her2 in each tumor sample was reported as a histoscore, calculated by the sum of the proportion score (0-5) and the intensity score (0-3) for a range between 0 and 8, with a maximum possible score of 8 [25].

#### Results

##### Isolation of tumorspheres from human breast core biopsies

The purpose of this study was to establish a reproducible method for the isolation of primary tumorspheres from patient core biopsies and characterization of subsequent tumor formation upon transplantation into nude mice. The establishment of a hetero-transplantation model, as described in this report, provides an improved and translatable murine model for the study of human breast cancer metastasis. Patient core biopsies were obtained using a 14-gauge spring-loaded gun, yielding 15-20 mg of tissue per biopsy. Tumorspheres were derived from patient core biopsies under serum-free, non-adherent culture conditions as described in Materials and Methods. The ER/PR/Her2 status differed in the patient samples (Table 1). The majority of patient samples were Grade 2 or higher and diagnosed as invasive ductal carcinoma (IDC) (Table 1). Tumorspheres were successfully isolated from all patient samples and ranged

**Table 1 Formation of primary tumor and metastasis in NUDE mice implanted with tumorspheres isolated from human breast core biopsies.**

Sample	Formation in mice	Latency to palpable tumor formation	Passage in Mice	Metastasis	Metastatic Latency	Age	Diagnosis	Grade	ER/PR/Her2 Status
4	0/2	N/A	N/A	Not Determined	N/A	56 years	IDC	Grade 2	ER <sup>+</sup> /PR <sup>+</sup> /Her2 <sup>-</sup>
5	5/6	74 days	Yes	Yes	254 days	44 years	IDC	Grade 3	ER <sup>+</sup> /PR <sup>+</sup> /Her2 <sup>-</sup>
6	6/6	47 days	Yes	Yes	232 days	62 years	IDC with lymphovascular invasion	Grade 2	ER <sup>+</sup> /PR <sup>+</sup> /Her2 <sup>+</sup>
7	5/6	72 days	Yes	Yes	214 days	77 years	IDC	Grade 2	ER <sup>+</sup> /PR <sup>+</sup> /Her2 <sup>-</sup>
8	4/6	35 days	Yes	Yes	248 days	63 years	IDC	Grade 2	ER <sup>+</sup> /PR <sup>+</sup> /Her2 <sup>-</sup>
9	6/6	46 days	Yes	Yes	279 days	66 years	IDC	Grade 1	ER <sup>+</sup> /PR <sup>+</sup> /Her2 <sup>+</sup>

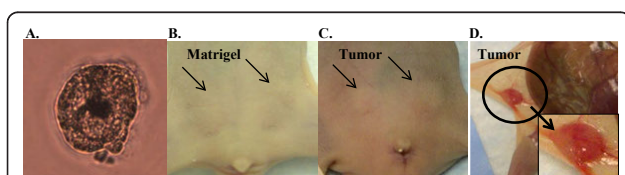
Tumor formation in NUDE mice following bilateral injections into the mammary fat pad of tumorspheres that were derived from the original patient biopsy. The latency or palpable tumor formation is indicated in number of days post-injection of tumorspheres into the mammary fat pad. 'Passage in mice' indicates that the primary tumor could be serially transplanted into mice to form subsequent primary tumors following *in vitro* formation of tumorspheres prior to injection into the mammary fat pad. Metastasis was determined by detection of human chromosome 17 by PCR using DNA isolated from mouse organs collected from mice injected with tumorspheres into the mammary fat pad. Metastatic latency is the average number of days between the injection of tumorspheres into the mammary fat pad and the detection of metastatic lesions in all organs analyzed by H+E staining

in size from 30  $\mu$ m to 100  $\mu$ m (Figure 1A). The isolated tumorspheres demonstrated a cell surface marker phenotype CD44<sup>+</sup>/CD24<sup>med/low</sup>/ESA<sup>+</sup> by immunocytochemistry (Additional file 1: Figure S1a-b), a phenotype previously determined to be tumorigenic in immune-deficient mice [27].

#### Tumor formation and serial transplantation in NUDE mice

To establish the presence of tumor-initiating cells within the isolated tumorspheres, cells were injected into the mammary fat pad of female NUDE mice in combination with Matrigel and mice were monitored for tumor formation. Since previous *in vitro* experiments suggested dissociation of the tumorspheres caused decreased viability of the cells (data not shown), non-dissociated tumorspheres were injected into the mammary fat pad. Based on cell counts performed during previous *in vitro*

experiments, an estimate of 50-100 cells comprised a tumorsphere of 100  $\mu$ m in diameter. All of the primary tumor samples stained with H+E were histologically evaluated by a pathologist (K.M.). The tumor formation capabilities of tumorspheres isolated from samples 1-3 were conducted in NOD/SCID female mice (data not shown). Tumor formation for samples 1-3 was not observed 3 months post-injection and extension of the experiment was terminated because of a high incidence of thymic masses in the NOD/SCID mice that a previous study described as lymphoma development [28]. Consequently, subsequent experiments were performed with the mouse strain to Nu/Nu (NUDE). Injection of Matrigel alone into the mammary fat pad of NUDE mice did not result in tumor formation (Figure 1B, arrows). Injection of 50 tumorspheres (estimated total cells injected:  $1-5 \times 10^3$  cells) isolated from samples 5-9 in combination with Matrigel into the mammary fat pad resulted in formation of small, palpable tumors within 3 months post-injection (Figure 1C-D, arrows) that were maintained until the end of the experiment (approx. 9-12 months post-injection). Tumorspheres isolated from sample 4 did not form tumors when injected into the mammary fat pad in combination with Matrigel (Table 1). Tumorspheres were re-isolated from the tumors formed in the mammary fat pad by employing the same serum-free, non-adherent culture conditions used to isolate tumorspheres from patient core biopsies. Serial transplantation was demonstrated by the injection of re-isolated tumorspheres into the mammary fat pad of NUDE mice in combination with Matrigel. Samples 5-9 were serially transplanted in this manner three times through NUDE mice. In summary, the *in vitro* selection



**Figure 1 Tumor formation in the mammary fat pad upon injection of human tumorspheres.** A. Light microscopy of a representative tumorsphere isolated from a patient core biopsy following *in vitro* culture for 10 days. B. Injection of Matrigel alone into the 3rd mammary fat pad of female NUDE mice. C, D.  $\leq 5 \times 10^3$  cells derived from the original patient biopsy were injected into the 3rd mammary fat pad in the form of 'tumorspheres' (with Matrigel) and resulted in formation of small, palpable tumors within 3 months post-injection with an approximate, sustainable tumor volume of 100 mm<sup>3</sup>.



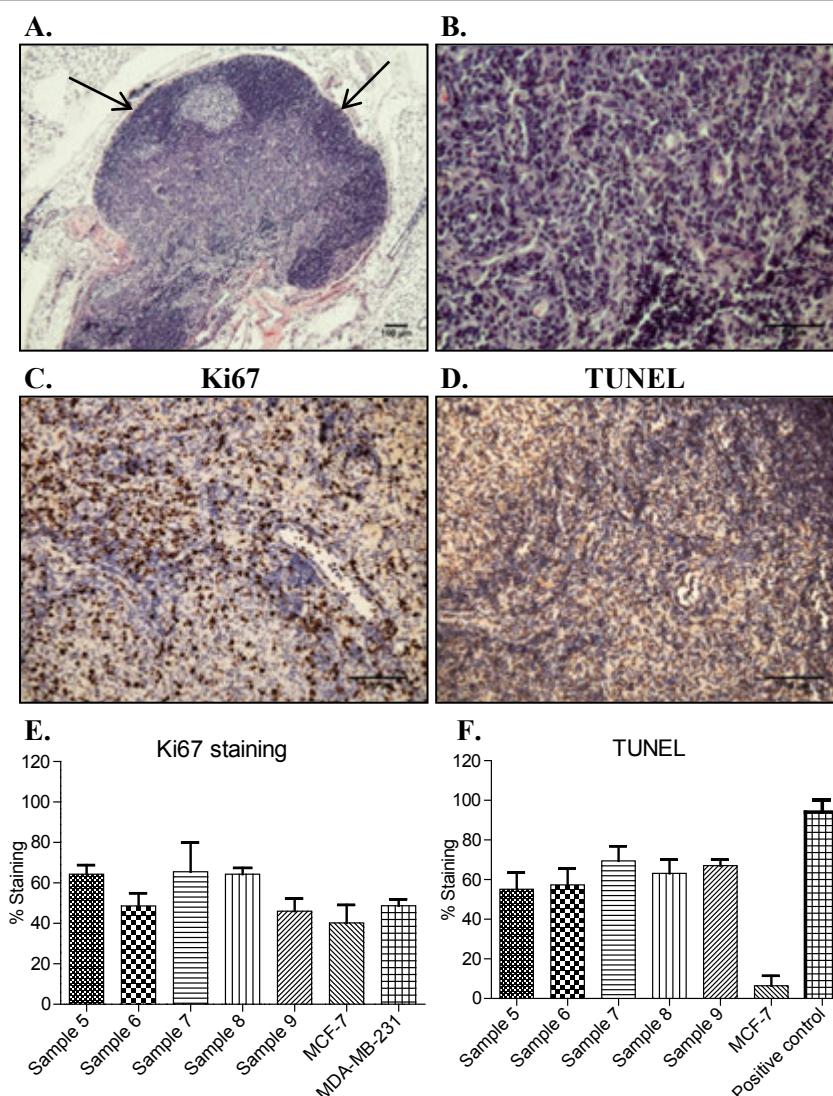
for tumorspheres prepared from human breast core biopsies contained bTICs that formed small tumors when injected into the mammary fat pad of NUDE mice. These tumors were serially transplantable through NUDE mice upon re-isolation of tumorspheres *in vitro* and re-injection of tumorspheres into the mammary fat pad of mice.

#### **Characterization of tumors formed in the mammary fat pad upon injection of tumorspheres**

H+E staining was performed on sections of the primary tumors removed from the mammary fat pad. The edges of the tumors were often occupied by a dense population of cells as compared to areas closer to the center of the tumor that were less dense (Figure 2A, arrows). Tumors consisted of small tumor cells with pleomorphic nuclei that did not exhibit tubule formation (Figure 2A-B). IHC was performed on sections of the tumors to determine the number of proliferating cells using a rabbit monoclonal ki67 antibody. Over 40% of the cells were proliferating in sample 6 and 9, and over 60% of the cells were proliferating in samples 5, 7, and 8 (Figure 2C, E). To understand how a small tumor size could be maintained in the context of significant proliferation, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed on sections of the tumors to determine the rate of apoptosis. The rates of apoptosis for samples 5-9 were similar to the rates of proliferation for each sample (Figure 2D, F) indicating a large degree of cell turnover in the tumors. In contrast, rapidly growing MCF-7 breast tumor xenografts did not display significant apoptosis (Figure 2F). A section from an MDA-MB-231 breast tumor xenograft incubated with 4 U/ml of DNase I at 37°C for 10 min was used as a positive control (Figure 2F, positive control).

The tumors were analyzed for the expression of a wide range of markers by IHC. Sections of MCF-7 and MDA-MB-231 tumor xenografts were used as controls for positive and negative staining by IHC for all antibodies (Additional file 2: Figure S2a-l). Expression levels for each marker were quantified using the MachBiophotonics ImageJ 1.42I program (as described in the Materials and Methods). All tumor samples were negative for estrogen receptor  $\alpha$  (ER $\alpha$ ) and the mesenchymal marker vimentin (data not shown). The cell adherens junction protein E-cadherin is normally expressed in the membrane of differentiated epithelial cells and more differentiated breast cancer cells. E-cadherin was observed at variable levels in the cytoplasm and nucleus in all tumor samples but not within the cell membrane (Figure 3A, H).  $\beta$ -catenin, a central mediator of the WNT pathway, binds to E-cadherin at the membrane in conjunction with a complex of proteins connecting the adherens

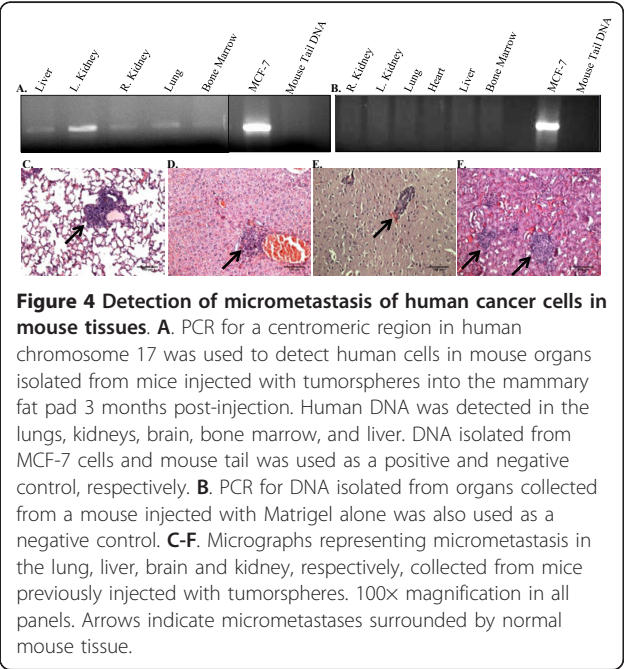
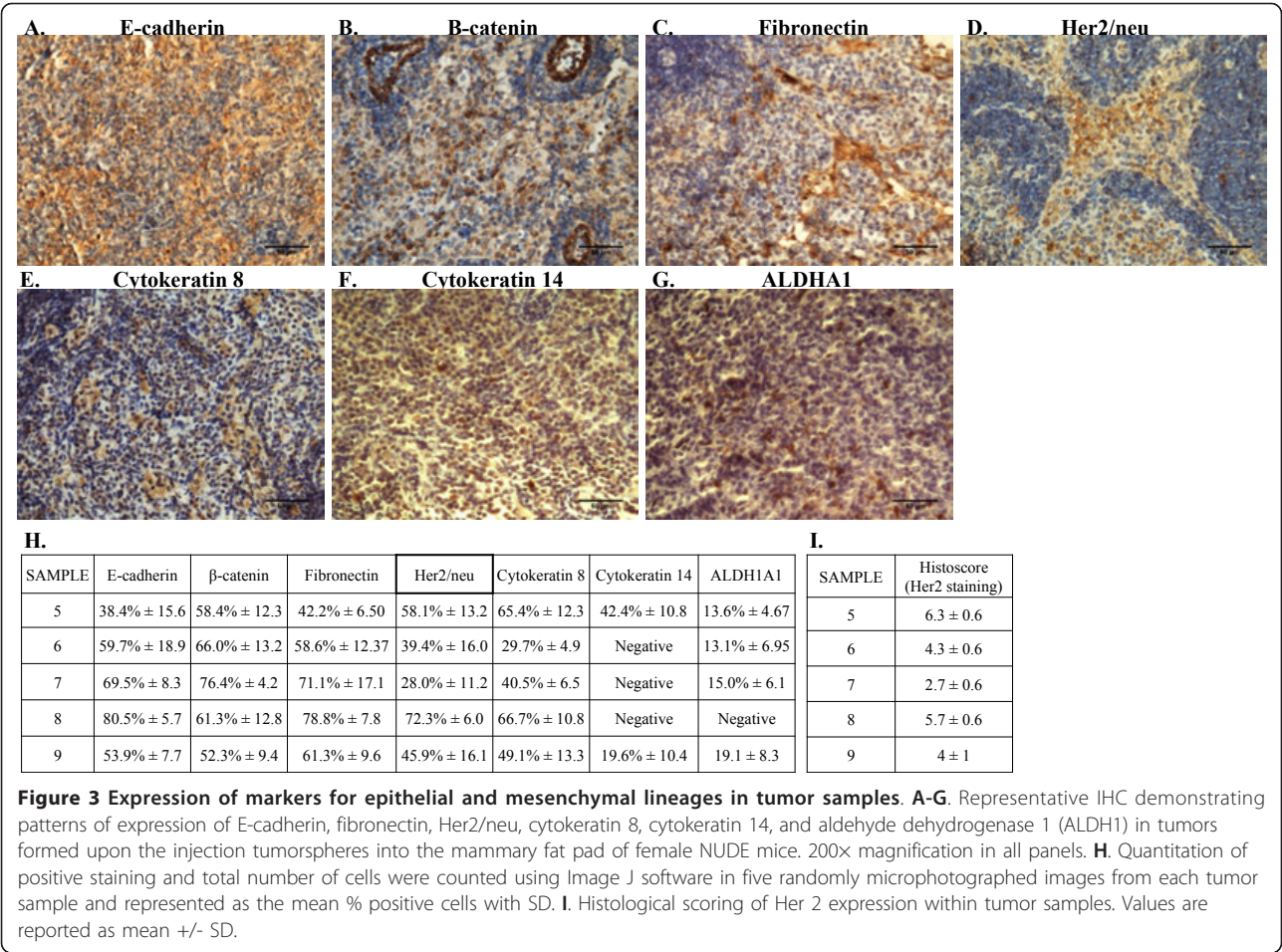
junction to components of the cytoskeleton [29,30]. In the absence of membrane E-cadherin,  $\beta$ -catenin is either rapidly degraded or can translocate to the nucleus upon activation of WNT signaling. In corroboration with the aberrant cytoplasmic and nuclear E-cadherin staining, localization of  $\beta$ -catenin was also observed at variable levels in the cytoplasm and nucleus of all the tumor samples (Figure 3B, H). Although all the tumor samples were negative for vimentin, another mesenchymal marker fibronectin was detected in all samples (Figure 3C, H). Her2/neu was detected by IHC in all tumor samples however the extent of expression was highly variable between samples (Figure 3D, H). In addition to quantification using the MachBiophotonics ImageJ 1.42I program, Her2/neu expression within the tumor samples was expressed as a histoscore (as described in the Methods) (Figure 3I). Based on the reported histoscores, sample 7 exhibited the lowest expression of Her2 whereas sample 5 exhibited the highest expression of Her2 as compared to the other samples (Figure 3I). The detection of Her2/neu staining by IHC in the experimental tumors is not equivalent to the clinical diagnosis of Her2/neu positive tumors, which is based predominantly upon Her2/neu gene amplification. All tumors showed positive staining using an antibody to broad-spectrum cytokeratins at variable levels between tumors (data not shown) indicating the presence of epithelial lineage cells in the tumor. IHC for cytokeratin 8 and cytokeratin 14 was performed to determine the presence of luminal and myoepithelial cell lineages, respectively, within the tumors. Cytokeratin 8 was detected in all samples at variable levels (Figure 3E, H) whereas cytokeratin 14 was only detected in sample 5 and 9 (Figure 3F, H). These data indicate that tumors were comprised of mixed luminal and myoepithelial lineage tumor cells with some tumors negative for myoepithelial lineage tumor cells. Recently, aldehyde dehydrogenase (ALDH) has been implicated as a stem cell marker for both normal mammary cells and breast cancer cells [31,32]. IHC using a rabbit monoclonal antibody against ALDH1A1 demonstrated less than 20% of cells in all tumors expressed ALDH1A1 and no expression was detected in tumors formed from sample 8 (Figure 3G, H). These data, along with the cytokeratin staining, indicate that the tumors formed upon injection of tumorspheres into the mammary fat pad of NUDE mice did not entirely retain the primitive features of the tumorsphere [16,33], but instead exhibited marked heterogeneity in expression of lineage specific epithelial and mesenchymal markers. To confirm that tumors contained cells of human origin, IHC was performed using a mouse monoclonal antibody against human nuclear antigen (HNA) [34,35]. Sections of a human MDA-MB-231 breast tumor xenograft was used as a positive control for HNA staining



**Figure 2 Morphology, proliferation and apoptosis within primary tumors formed upon injection of tumorspheres.** **A, B.** H+E staining of 5  $\mu$ m paraffin-embedded tumor sections derived from patient Sample 5 at 40x and 200x, respectively. **C, E.** IHC and quantitation for Ki67 were performed on 5  $\mu$ m paraffin-embedded tumor sections derived from patients Samples 5-9. Ki67 positive cells and total number of cells were counted using Image J software in five randomly microphotographed images from each tumor sample and represented as the mean % positive cells with SD. **D, F.** TUNEL staining and quantitation (as described above) for apoptosis. MDA-MB-231 human breast cancer xenograft incubated with 4 U/ml DNase I was used as a positive control for TUNEL. Values are reported as mean  $\pm$  SD.

(Additional file 3: Figure S3a); as a negative control, PBS used in place of the HNA primary antibody step for staining tumors formed upon injection of tumorspheres (Additional file 3: Figure S3b); sections of a mouse kidney incubated with the HNA antibody from a non-tumor bearing animal was also used as a negative control (Additional file 3: Figure S3c). In all tumors (samples 5-9), a majority of the cells stained positive for HNA as shown in the representative micrograph for sample 6 (Additional file 3: Figure S3d). Metastatic lesions within the liver and lung were also positive for HNA (Additional file 3: Figure S3e and f, respectively).

**Metastatic human cancer cells detected in mouse tissues**  
Micrometastasis of human cancer cells to mouse kidney, liver, lung, brain and femurs (bone marrow) was assessed at 3 months post-injection using PCR to detect human chromosome 17 in the mouse tissues. Although no visual macrometastatic lesions were observed within any of the organs at 3 months post-injection, human DNA was detected in the kidneys, liver, lung, bone marrow (Figure 4A), and brain (data not shown). As a negative control, DNA was isolated from the organs of a mouse injected with Matrigel alone into the mammary fat pad; no signal was detected (Figure 4B). H+E staining



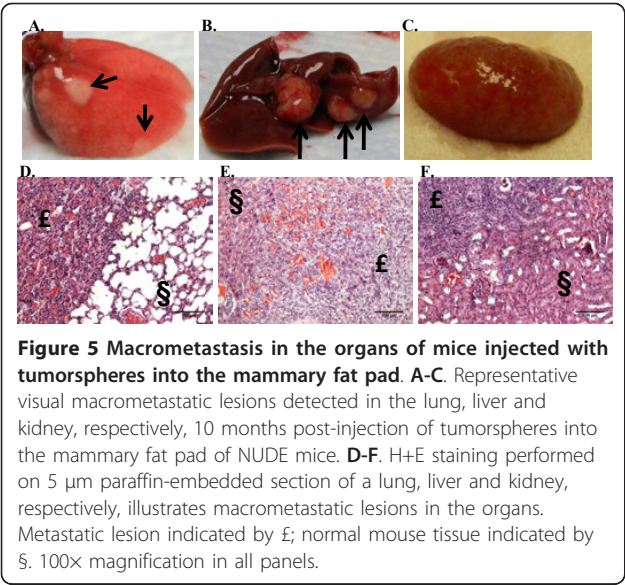
demonstrated micrometastases within the lung, liver, brain and kidney (Figure 4C-F, respectively).

Data from Figure 4 demonstrated that by 3 months post-injection of tumorspheres into the mammary fat pad, tumor cells had disseminated to distant organs. By 8 months post-injection, macrometastatic (visual) lesions were observed in the lung, liver and kidney for samples 5-9 (Figure 5A-C, respectively). H+E staining performed on sections of organs with visual metastatic lesions at the time of necropsy confirmed the presence of large metastatic lesions within the lung, liver and kidneys (Figure 5D-F, respectively).

**Organ tropism of the metastatic cells and the metastatic burden within the mouse organs**

Paraffin-embedded sections of lungs, livers, kidneys and brains from samples 5-9 were stained with H+E to further determine the metastatic potential of the tumorspheres injected into the mammary fat pad. Since tumorspheres isolated from sample 1-3 did not form tumors in the mammary fat pad of NOD/SCID mice, the metastatic potential for these samples was not

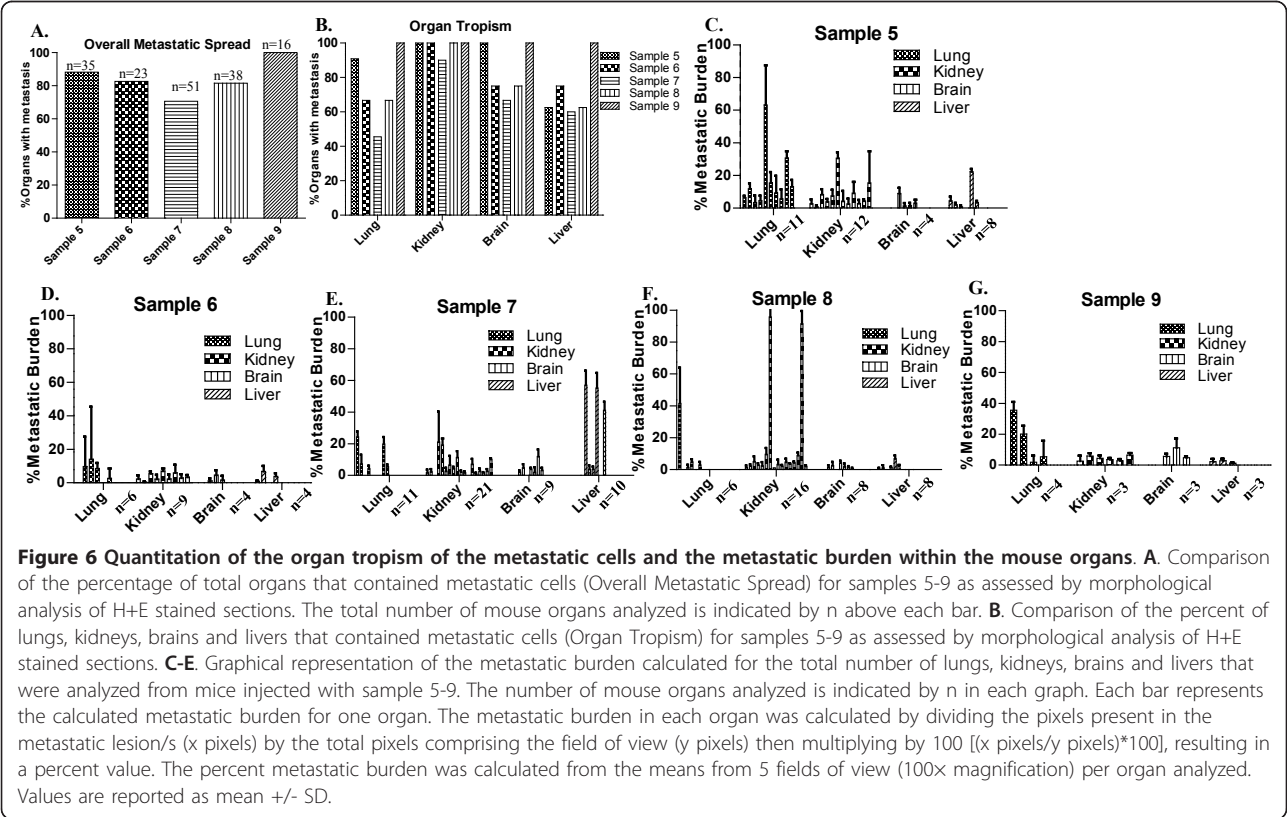




determined. A comprehensive analysis of metastasis was performed to: 1) determine the percentage of all organs examined (lung, kidney, brain, liver) that exhibited metastases for each of samples 5-9 (overall metastatic spread); 2) compare tropism of each sample to different organs; and; 3) quantitate the relative metastatic burden

within each organ for each sample as a measure of the ability of metastatic cells to colonize organ sites and grow into larger lesions. The total number of organs with detectable metastases was counted to determine the overall metastatic spread for each sample. The data was further separated by the particular organ with detectable metastases (lung, liver, kidney and brain) to determine tropism. The metastatic burden within each organ was then quantified as described in the material and methods. The number (n) of lungs, kidneys, brains and livers analyzed for each sample is indicated in Figure 6C-G.

The percentage of all organs examined with metastasis (overall metastatic spread), without regard to the size of the metastatic lesions, was comparable between all samples (Figure 6A). Sample 7 demonstrated the lowest (71%), and sample 9 demonstrated the highest (100%) overall metastatic spread. Analysis of the percentage of organs with metastasis (without regard for the size of the metastatic lesions) revealed qualitative differences in the organ tropisms of the samples. Samples 5 and 9 exhibited the highest tropism to lung and brain as compared to the other samples and sample 9 also exhibited higher tropism to liver (100%) than any other sample (Figure 6B). Sample 5 additionally demonstrated the largest % metastatic burden in the lung compared to other





tumor samples (compare lung % metastatic burden in Figure 6C to Figures 6D-G). Although sample 9 demonstrated the highest tropism to liver (Figure 6B), the metastatic burden in the liver did not exceed 10% (Figure 6G) indicating that although sample 9 metastasized to liver in 100% of animals, the metastatic tumor cells did not develop into large metastatic lesions. In contrast, although sample 7 showed the lowest tropism to liver compared to other samples (60%, Figure 6B), the metastasized tumor cells yielded the greatest metastatic burden in the liver compared to other samples (Figure 6E). Sample 7 demonstrated the lowest tropism to the brain (Figure 6B) although the metastatic burden in the brain was comparable to the other samples (Figure 6E). Supplemental Figure 4 represents the % metastatic burden in each tissue for each sample as a function of the time the organs were removed after initial injection of tumorspheres into the mammary fat pad (Days post-injection). An increase in metastatic burden in any of the organs did not correlate with the number of days post-injection (Additional file 4: Figures S4a-e). However, sample 7 demonstrated larger metastatic lesions at earlier time points as compared to the other samples (Additional file 4: Figure S4c).

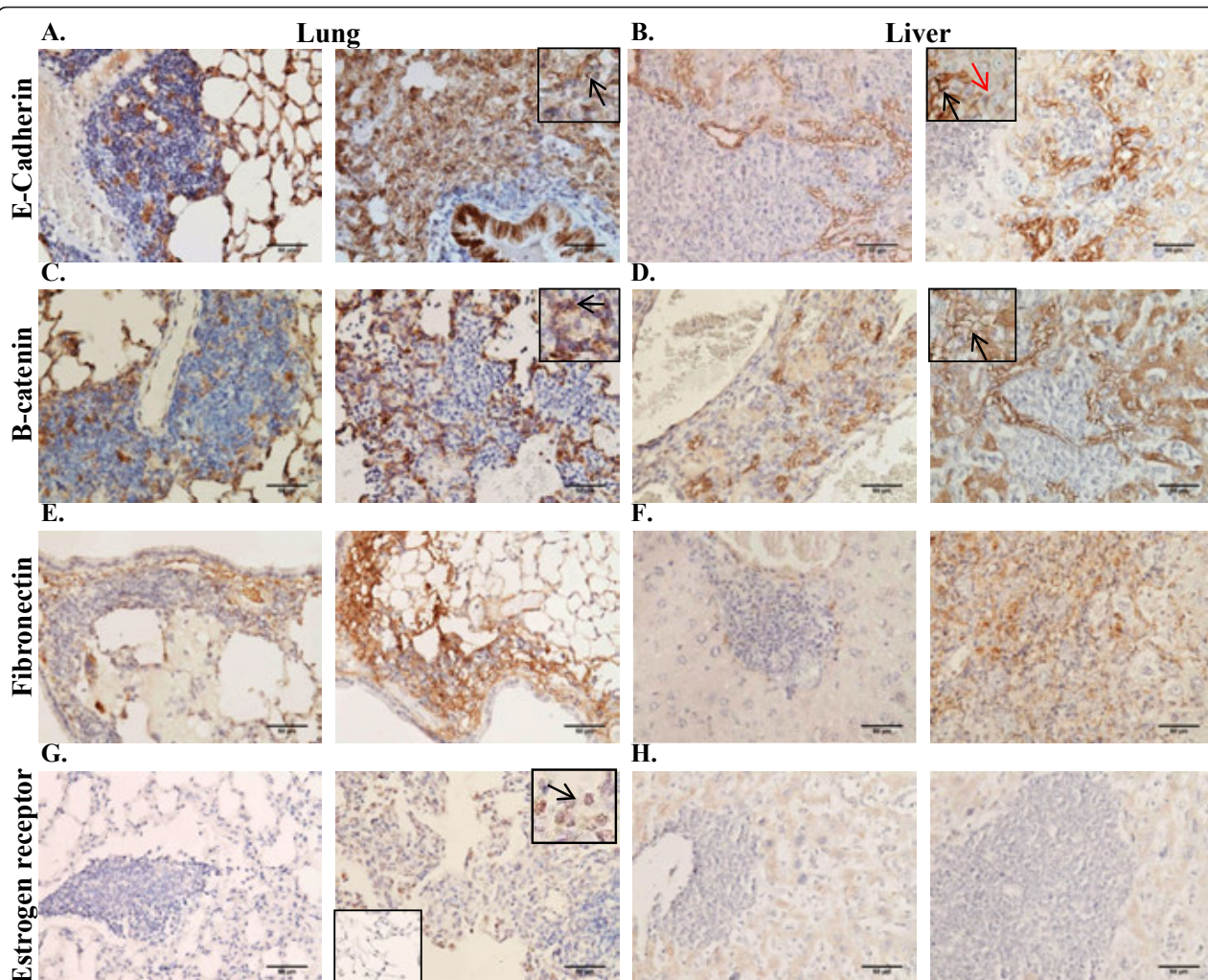
#### Characterization of the metastatic cells within the mouse organs

It is hypothesized that tumor cells acquire metastatic potential following an epithelial to mesenchymal transition (EMT) that permits local invasion and migration to distant metastatic sites. Once cells arrive at these metastatic sites, it is further hypothesized that tumor cells may undergo a reversion to reacquire epithelial characteristics that will permit survival and outgrowth at the ectopic site [30,36]. Therefore the expression of E-cadherin and  $\beta$ -catenin, two important modulators of EMT, was assessed by IHC within the metastatic lesions in the lung and liver. The localization of E-cadherin and  $\beta$ -catenin in the lung and liver was markedly different than the localization in the primary tumor (see Additional file 5: Table S1 for comprehensive comparison of marker expression profiles between primary tumor and metastatic lesions). In contrast to the predominantly cytoplasmic and nuclear localization of E-cadherin in the primary tumors, E-cadherin was detected in the membrane of the metastatic cells in the lung and liver although not all metastatic cells demonstrated E-cadherin staining (Figure 7A and 7B). In the liver, E-cadherin expression was most consistently observed in metastatic cells within close proximity to resident hepatocytes (Figure 7B, black arrow). Interestingly, hepatocytes within close proximity to metastatic cancer cells demonstrated stronger expression of E-cadherin in the

membrane as compared to hepatocytes not proximal to metastatic cancer cells (Figure 7B, red arrow). Similar to localization of E-cadherin,  $\beta$ -catenin expression was also detected predominantly in the membrane of the metastatic cells in the lung and the liver (Figure 7C and 7D) in contrast to the cytoplasmic and nuclear localization in the primary tumor. The pattern and intensity of  $\beta$ -catenin staining was similar to that of E-cadherin within the same lung tissue samples (Figure 7C, black arrow).  $\beta$ -catenin was most strongly expressed in the membrane of metastatic cells within close proximity to hepatocytes in the liver, similar to the results with E-cadherin (Figure 7D, black arrow). Fibronectin, a component of the extracellular matrix (ECM), exhibited variable expression within metastatic lesions in the lung and the liver (Figure 7E and 7F). All metastatic lesions in the liver did not express ER $\alpha$ , consistent with the lack of ER $\alpha$  expression in the primary tumors (Figure 7H). However, metastatic lesions in the lung of mice injected with tumorspheres isolated from samples 6 and 8 demonstrated heterogeneous re-expression of ER $\alpha$  (Figure 7G, arrow).

#### Discussion

Current research implies the presence of a population of cells in breast tumors with tumor-initiating capabilities. A number of cell surface markers have been used to sort for breast cancer cells with tumor-initiating capacity, including CD44 [6], CD133 [37], ALDH [31,38], CD90 [39,40], and CD117 (KIT) [41]. However, attempts to eliminate artifact may result in the biased selection of a small sub-population of cells present in disaggregated tissue. Presented in this study is an efficacious model for the *in vitro* isolation of tumorspheres, containing breast tumor-initiating cells, from human breast core biopsies. Injection of isolated tumorspheres into the mammary fat pad of NUDE mice resulted in formation and maintenance of small, palpable tumors that exhibited elevated proliferation and apoptosis. Within 8 months post-injection, widespread metastasis to mouse organs occurred most notably to liver, lung, and kidney. The cells within the tumors present in the mammary fat pad displayed heterogeneous expression of a range of markers for epithelial and mesenchymal differentiation that included cytokeratins, E-cadherin,  $\beta$ -catenin, fibronectin, as well as Her2/neu. Histological heterogeneity was observed both within individual tumors, and between tumors formed from tumorspheres isolated from different patient samples. Whereas primary tumors did not exhibit E-cadherin staining in cell membranes, a subset of tumor cells that had metastasized to the lung and the liver exhibited a re-distribution of E-cadherin to the membrane. These data describe a novel hetero-



**Figure 7 Expression of markers for epithelial and mesenchymal lineages in metastatic lesions of lung and liver.** A-B. IHC performed on 5  $\mu$ m paraffin-embedded sections of a lung and liver using a monoclonal anti-human antibody to E-cadherin. C-D. IHC performed on 5  $\mu$ m paraffin-embedded sections of a lung and liver using a polyclonal anti-human antibody to  $\beta$ -catenin. E-F. IHC performed on 5  $\mu$ m paraffin-embedded sections of a lung and liver using a polyclonal anti-human antibody to fibronectin. G-H. IHC performed on 5  $\mu$ m paraffin-embedded sections of a lung and liver using a monoclonal anti-human antibody to ER $\alpha$ . G. Inset panel (bottom left) demonstrates negative staining in normal lung cells in proximity to the metastatic lesion in the lung. 200x magnification in all panels.

transplantation tumor model that exhibits a metastatic profile that recapitulates the development of metastasis in human breast cancer patients.

Tumorspheres were successfully isolated from all breast core biopsies including samples 1-3, which were not included in the data presented due to the lack of tumor formation and development of thymic lymphoma in NOD/SCID mice (as described in the Results section). Samples 5-9 tumorspheres were derived from patients with invasive ductal carcinoma (IDC), similar to other reports investigating the tumorigenic potential of breast cancer cells isolated as tumorspheres *in vitro* [16,33]. Although FACS has been used to isolate tumorigenic breast cancer cells from other breast cancer subtypes

such as inflammatory breast cancer and lobular carcinoma [27], there are no reports demonstrating the successful isolation of tumor-initiating cells as tumorspheres from these breast cancer subtypes. When the host was changed to NUDE mice, the methods described herein reproducibly resulted in tumorsphere formation *in vitro*, and in tumor formation for all samples derived from primary breast core biopsies irrespective of ER/PR/Her2 status, tumor grade or stage. Tumorsphere number and size varied between samples, however differences could not be correlated with ER/PR/Her2 status, tumor stage or grade.

Tumorspheres isolated under select culture conditions likely contain a heterogeneous population of cells most

of which will not likely have tumor-initiating capabilities. Therefore tumor formation upon the injection of a minimal number of cells that were not selected on the basis of epithelial origin or differentiation potential indicated the presence of tumor-initiating breast cancer cells within the tumorspheres. The injection of non-disaggregated tumorspheres, as opposed to single cell suspensions, establishes a novel method for the investigation of tumorigenicity *in vivo*. The absence of mechanical and/or enzymatic stress during preparation of the cells for injection improved the tumor cell viability. Therefore the injection of non-dissociated tumorspheres, in combination with the immune-deficient mouse model employed (NUDE mouse strain), contributed to the high tumor engraftment rates observed. However, future studies should determine differences in tumor formation and metastatic potential between single cell suspensions and non-disaggregated tumorspheres. Differences detected may reveal the effects of cell selection bias based on methodologies employed when preparing cells for injection.

The small primary tumor volume observed in the mammary fat pad suggested a low level of tumor cell proliferation. However the number of proliferating cells detected by Ki67 staining was high in all tumor samples. This high proliferation was offset by a number of cells undergoing apoptosis. This equilibrium between proliferation and apoptosis provided an explanation for the formation and long-term propagation of the small, palpable tumors, indicating the tumors were persisting in a state of tumor dormancy. However it also raised the question as to why the high level of proliferation observed in the tumors did not eventually overcome the apoptosis and result in larger tumor volumes with time. One possible explanation could be the inability of tumors to initiate neoangiogenesis that was manifest in high rates of apoptosis and proliferation that culminated with tumor dormancy [42,43]. Failure of the injected cells to efficiently recruit mouse stromal cells/endothelial cells would lead to nutrient and oxygen deprivation that would result in high levels of apoptosis to compensate for a high proliferation of the tumor cells. Alternatively, this disruption in homeostasis may occur as a later event during tumor progression, upon sufficient acquisition of somatic mutations within the differentiated progeny. Nonetheless, the metastatic phenotype demonstrated in this study implies that metastasis is an early event in tumorigenesis; conferring implications that challenge the linearity of breast tumor progression.

Traditional xenograft models derived from established breast cancer cell lines (e.g. MCF-7, MDA-MB-231) display fairly homogeneous organization, morphology, and protein expression patterns. In contrast, characterization of the tumors derived from primary tumorspheres

presented here revealed heterogeneity among samples in morphology and marker expression. Because the tumorspheres exhibited stem-like properties, it might be expected that tumors in mice derived from the tumorspheres would exhibit similar morphology as the patient tumors. Because the tumorspheres exhibited stem-like properties, it might be expected that tumors in mice derived from the tumorspheres would exhibit similar morphology as the patient tumors. Although the human breast tumor samples ranged from grade 1-3, the tumors in the mammary fat pad did not exhibit all of the morphological characteristics of the patient tumors, such as tubule formation and ER and Her2 expression. This discrepancy is likely a result of the microenvironment within the mammary fat pad that is mostly devoid of the stromal and cellular components present in human mammary tissue, and instead is composed predominantly of adipose tissue. It is possible that the inclusion of human stromal/cellular components with the tumorspheres would more accurately recapitulate the microenvironment within human mammary tissue resulting in tumors that might exhibit tubule formation, ER/PR expression and other characteristics of low grade, more differentiated. In general, the edges of the tumors were lined with dense areas of cells that were also observed within the tumors as a dense ring of cells surrounding a more diffuse distribution of tumor cells (Figure 2). Although the expression patterns of most markers used in the characterization of the tumors did not correspond to this evident cellular organization, the expression of  $\beta$ -catenin did correlate with this organization displaying strong expression in the ring of cells encapsulating the diffuse population of cancer cells. All tumors in the mammary fat pad were negative for ER $\alpha$  despite the varied ER $\alpha$  status of the patient samples. During an EMT in breast cancer, E-cadherin expression in the membrane is reported to be lost or re-localized to the cytoplasm [29,44]. Consistent with a mesenchymal phenotype, in the primary tumors E-cadherin and  $\beta$ -catenin expression was localized in the cytoplasm and nucleus (Figure 3). Given that loss of E-cadherin expression in breast cancer is associated with an EMT and with the metastatic process, the unique expression pattern of E-cadherin in the present tumor model that exhibits a metastatic phenotype warrants further study.

Human metastatic cancer cells were detected by PCR at 3 months post-injection of tumorspheres into the mammary fat pad within the lung, liver, kidney, brain and femur (Figure 4). However the development of large metastatic lesions was observed by H+E staining in the lung, kidney and liver only. Comparison of the organ-specific percent metastasis to the metastatic burden within each organ revealed interesting results. Although sample 7 demonstrated the lowest percent metastasis



overall and within the organs analyzed, the percent metastatic burden within each organ was comparable to or more often higher on average as compared to the other samples. In contrast, sample 9 demonstrated the highest percent metastasis overall and within the organs analyzed, however the percent metastatic burden within each organ was minimal compared to the other samples. These conflicting results elucidate a potential shortcoming in the current methods employed for predicting metastatic disease. The ability of cancer cells to metastasize from the primary site to distant sites may not accurately reflect the actual metastatic potential of the cells. Rather, understanding and predicting the adaptation of the cells that have arrived at the distant site of metastasis, may more accurately determine the potential for the development of overt metastatic disease. Samples 5 and 9, the only tumor samples derived from patient biopsies with clinical diagnosis of triple negative (ER<sup>-</sup>/PR<sup>-</sup>/Her2<sup>-</sup>), demonstrated the highest average metastatic burden within the organs. This observation may imply a growth advantage at distant sites for metastatic cells derived from triple negative tumors, although additional samples would be needed to demonstrate statistical significance. Despite the detection of metastasis by H+E staining in 60%-100% of brains analyzed, the metastatic burden was on average between only 1-3%. The blood-brain barrier (BBB) consists of tight junctions and adherens junctions between the brain endothelial cells, restricting the passage of substances from the bloodstream into the brain [45]. Impedance of entry into the brain by the BBB may contribute to the apparent extended dormancy period of the metastatic cells present in the brain, in combination with other influencing factors. Overall, the long duration from the time of detection of cancer cells in the organs (by PCR and/or histological staining) to the development of visual macrometastasis in mouse organs was similar to the delay in development of measurable metastasis in breast cancer patients following diagnosis of primary breast tumors [46,47]. The extended latency between the injection of tumorspheres and the development of macroscopic metastatic lesions is a limitation of this model. The injection of a higher number of cells may increase the time to tumor formation and the tumor volume in the mammary fat pad, however we speculate that the observed metastatic latency would not be affected. The organ microenvironment and intrinsic properties of the disseminated cells likely predominantly contribute to the observed metastatic latency. However secreted factors from the primary tumor within the mammary fat pad could also influence metastatic progression, therefore an increase in tumor volume may affect the proliferative state of disseminated tumor cells at distant sites. Future studies investigating the effects of larger tumor volumes on metastatic latency, possibly by

the injection of increasing numbers of cells into the mammary fat pad, may elucidate the influence of secreted factors from the primary tumor on disseminated cancer cells. The detection of micrometastases in an array of organs with the development of macrometastases in only a select few of those organs suggests that mechanical/stochastic forces may permit entry of cancer cells into a range of organs; however metastatic cells will only survive and develop into overt lesions when present within a permissive, conditioned metastatic niche [20].

Whereas cytoplasmic and nuclear localization of E-cadherin and  $\beta$ -catenin was observed in the primary tumors, E-cadherin and  $\beta$ -catenin were re-expressed in the membrane in a subset of metastatic cells in the lung suggesting a possible reversion back to an epithelial phenotype (a mesenchymal to epithelial transition) [36,48]. E-cadherin and  $\beta$ -catenin expression was predominantly observed in the membrane of metastatic cells proximal to hepatocytes in the liver. Furthermore, hepatocytes proximal to metastatic cancer cells demonstrated stronger E-cadherin expression as compared to hepatocytes not within close proximity to metastatic cancer cells. Chao et al. demonstrated re-expression of E-cadherin in the membrane of MDA-MB-231 breast carcinoma cells *in vivo* and *in vitro* when in close proximity to hepatocytes [49]. Additionally, in prostate cancer models of metastasis to the liver, E-cadherin was shown to accumulate at the interface with hepatocytes [50,51]. These findings further support the importance of crosstalk between the cancer cells and native cells present within the organ. Fibronectin, a component of the extracellular matrix (ECM), plays an important role in cell migration, adhesion, maintenance of cell shape and wound healing. Fibronectin-dependent signaling has been linked to cancer cell dormancy, involved in the quiescent to proliferative switch [52,53]. Many of the smaller metastatic lesions in the liver did not demonstrate expression of fibronectin, however fibronectin was found variably expressed within the larger metastatic lesions. This observation could implicate fibronectin signaling as a possible mechanism involved in the development of macrometastatic lesions within the liver. The metastatic lesions within the liver were negative for ER $\alpha$  in all samples, consistent with the lack of expression within primary tumors. However in samples 6 and 8, metastatic lesions in the lungs of mice bearing primary tumors demonstrated variable re-expression of ER $\alpha$ , whereas the normal lung tissue did not exhibit expression of ER $\alpha$ . Interestingly, samples 6 and 8 were derived from patient biopsies clinically diagnosed as ER $\alpha$  positive. Previous clinical studies have reported differences in the expression of ER $\alpha$  between the primary patient tumor and the metastatic sites [54,55] although the underlying

mechanisms to explain the altered expression are unknown. These results reinforce the importance of the microenvironment within the metastatic niche for influence on the differentiation state of the metastasized tumor cells and the expression of clinically relevant markers. The heterogeneity of the metastatic cells within the lung and the liver implied plasticity, whether innate or induced, during the metastatic process. Collectively, these findings demonstrate the complex integration of extrinsic and intrinsic factors affecting the behavior and phenotype of breast cancer cells during the metastatic process.

The present study data demonstrates a novel model for the study of human breast cancer breast cancer metastasis using samples obtained directly from patient biopsies. The metastatic phenotype demonstrated upon injection of tumorspheres into the mammary fat pad permits the study of all steps within the metastatic process. In particular, the development of macrometastatic lesions with organ-specific phenotypic distinctions provides a superior model for the investigation of organ-specific effects on metastatic cancer cell survival and growth. This model accurately recapitulates the metastatic latency observed clinically, permitting the development of therapeutics that target metastatic cells during dormancy prior to activation. Experimental manipulation of the organ-specific microenvironment could reveal molecular targets that are clinically accessible and biologically relevant. Furthermore, this model can be used to develop improved methods for the detection of micrometastatic cells and the prediction of metastatic disease.

## Conclusions

Primary breast tumor-initiating cells can be isolated as tumorspheres under non-adherent, serum free culture conditions from patient core biopsies independent of assigned grade or ER/PR/Her2 status. Isolated tumorspheres were tumorigenic in NUDE mice and had the capacity to metastasize from the primary site (i.e. mammary fat pad) to distant organs, such as the liver, lung, kidney, brain, and femur. Tumor cells at the metastatic sites exhibited organ-specific phenotypes that demonstrated plasticity of the metastatic cells dependent upon the organ microenvironment. This study describes a reproducible heterotransplant tumor model derived from patient biopsies that provides a novel method for the comprehensive study of breast cancer metastasis that better recapitulates the dormancy, complexity and heterogeneity within human breast cancer metastases.

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## Additional material

**Additional file 1: Figure S1. Characterization of cell surface marker expression of tumorspheres.** A. Immunocytochemistry (ICC) of tumorspheres prepared by formalin fixation and 5 µm paraffin-embedded sections using pre-conjugated antibodies against CD44-PE, and CD24-FITC. ICC for ESA-FITC was performed on tumorspheres prepared by centrifugation onto glass coverslips (cytospins). Tumorspheres demonstrate a CD44<sup>+</sup>/CD24<sup>low-med</sup>/ESA<sup>+</sup> cell surface marker phenotype. B. Isotype matched, pre-conjugated IgG control antibody mixture (IgG<sub>1</sub>-PE/IgG<sub>2a</sub>-FITC) was used as a negative control for ICC. 200x magnification in all panels.

**Additional file 2: Figure S2. MCF-7 and MDA-MB-231 breast tumor xenografts used as controls for IHC.** A-L IHC performed on 5 µm paraffin-embedded sections of MCF-7 (A-F) and MDA-MB-231 (G-L) xenografts using rabbit monoclonal E-cadherin antibody (A+G), rabbit polyclonal β-catenin antibody (B+H), rabbit polyclonal fibronectin antibody (C+I), rabbit monoclonal Her2/ErbB2 antibody (D+J), rabbit polyclonal cytokeratin 8 antibody (E+K), and rabbit monoclonal cytokeratin 14 antibody (F+L). IHC results on MCF-7 and MDA-MB-231 xenograft sections were used as positive and negative controls for the IHC results on tumors formed after injection of tumorspheres in the mammary fat pad (Figure 3). All panels 200x magnification.

**Additional file 3: Figure S3. Human nuclear antigen (HNA) staining detects human cells at in the primary tumor and at the metastatic sites.** A. 5 µm paraffin-embedded sections of MDA-MB-231 breast tumor xenograft used as a positive control for HNA (mouse anti-human nuclei monoclonal antibody) staining. B. Tumor sample matched negative control, with the replacement of the primary antibody with 1x PBS. C. Kidney isolated from a non-injected NUDE mouse, incubated with HNA to demonstrate human specificity with the lack of nuclear staining of the mouse kidney cells. D. Cells stain positive for HNA in 5 µm paraffin-embedded sections of a tumor removed from the mammary fat pad after injection of tumorspheres. E-F. HNA staining of 5 µm paraffin-embedded sections of metastatic lesions in the liver and lung, respectively confirms the human origin of the lesion, with the majority of nuclei staining positive. All panels 200x magnification.

**Additional file 4: Figure S4. Correlation between metastatic burden and the time after injection of tumorspheres that mouse organs were removed.** Graphical representation of the percent metastatic burden, previously calculated as described in Figure 6, for each tissue for each sample as a function of the time the organs were removed after initial injection of tumorspheres into the mammary fat pad (Days post-injection). Values are reported as mean +/- SD.

**Additional file 5: Table S1. Summary of heterogeneous marker expression between primary tumor (mammary fat pad) and metastatic lesions.** Tabular representation of the expression of E-cadherin, β-catenin, fibronectin, and ERα between samples in the primary tumor (mammary fat pad), metastatic lesions in the lung and the liver. Cytoplasmic localization is expressed as 'cyto' in the table. 'Variable' indicates the variability of staining within metastatic lesions.

## Abbreviations

BCSC: Breast cancer stem cell; ER: Estrogen receptor; bTIC: Breast tumor-initiating cell; HBSS: Hanks buffered saline solution; BSA: Bovine serum albumin; EGF: Epidermal growth factor; bFGF: Basic fibroblast growth factor; ESA: Epithelial specific antigen; PBS: Phosphate buffered saline; IHC: Immunohistochemistry; IDC: Invasive ductal carcinoma; H&E: Hematoxylin and eosin; TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; ALDH: Aldehyde dehydrogenase; HNA: Human nuclear antigen; EMT: Epithelial to mesenchymal transition; ECM: Extracellular matrix.

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# Authors' contributions

CGM carried out the study design, animal necropsy, data collection, statistical analysis, data interpretation, manuscript preparation, and literature search. MJW provided the patient core biopsies and collected the consent forms from the patients. LC performed mouse injections. KM provided histological analysis of tissues. RP participated in study design and subsequent analysis. BGR organized the study as the director, manuscript preparation, and funding the collection. All authors read and approved the final manuscript.

# Competing interests

The authors declare that they have no competing interests.

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# Disseminated Breast Cancer Cells Acquire a Highly Malignant and Aggressive Metastatic Phenotype during Metastatic Latency in the Bone

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## Abstract

**Background:** Disseminated tumor cells (DTCs) in the bone marrow may exist in a dormant state for extended periods of time, maintaining the ability to proliferate upon activation, engraft at new sites, and form detectable metastases. However, understanding of the behavior and biology of dormant breast cancer cells in the bone marrow niche remains limited, as well as their potential involvement in tumor recurrence and metastasis. Therefore, the purpose of this study was to investigate the tumorigenicity and metastatic potential of dormant disseminated breast cancer cells (prior to activation) in the bone marrow.

**Methodology/Principal Findings:** Total bone marrow, isolated from mice previously injected with tumorspheres into the mammary fat pad, was injected into the mammary fat pad of NUDE mice. As a negative control, bone marrow isolated from non-injected mice was injected into the mammary fat pad of NUDE mice. The resultant tumors were analyzed by immunohistochemistry for expression of epithelial and mesenchymal markers. Mouse lungs, livers, and kidneys were analyzed by H+E staining to detect metastases. The injection of bone marrow isolated from mice previously injected with tumorspheres into the mammary fat pad, resulted in large tumor formation in the mammary fat pad 2 months post-injection. However, the injection of bone marrow isolated from non-injected mice did not result in tumor formation in the mammary fat pad. The DTC-derived tumors exhibited accelerated development of metastatic lesions within the lung, liver and kidney. The resultant tumors and the majority of metastatic lesions within the lung and liver exhibited a mesenchymal-like phenotype.

**Conclusions/Significance:** Dormant DTCs within the bone marrow are highly malignant upon injection into the mammary fat pad, with the accelerated development of metastatic lesions within the lung, liver and kidney. These results suggest the acquisition of a more aggressive phenotype of DTCs during metastatic latency within the bone marrow microenvironment.

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## Introduction

Once considered the final step during cancer progression, recent evidence implicates metastasis as an early event in breast cancer [1–4]. Disseminated tumor cells (DTCs) may be present at distant sites at the time of primary diagnosis of breast cancer in patients that exhibit no outward signs of clinical metastases. As a preferential site of metastasis for breast cancer [5], the detection of DTCs in the bone of breast cancer patients has become an important prognostic tool. It is estimated that DTCs can be detected in the bone marrow for up to 40% of breast cancer patients using the current detection technology [6,7]. As a strong independent prognosticator, patients with DTCs in the bone marrow have an overall worse prognosis, as well as a higher

propensity for local and distant relapse, compared to patients without DTCs in the bone marrow [8–10]. Despite the clinical significance of DTCs in the bone marrow, the biological relevance remains controversial [11].

Although DTCs can be detected in the bone marrow of early breast cancer patients, clinical manifestation of bone metastasis and/or recurrence often does not emerge for years or even decades after initial diagnosis [4,12]. The lag time between detection of DTCs and manifestation of disease indicates the cells have become dormant, persisting as viable but non-proliferating cells [4,13,14]. The mechanisms by which the cells enter a dormant state can be intrinsic, a result of genetic and/or epigenetic modifications, or as a consequence of the microenvironment in which the cells reside [15]. Studies have shown



significantly less chromosomal aberrations in DTCs in the bone marrow as compared to cells in the primary tumor [1,3,16] suggesting DTCs have not acquired the necessary genetic alterations to overcome growth restraints. However, early DTCs have also been shown to be genomically very unstable [17,18]. These conflicting reports concerning the intrinsic properties of DTCs indicates it is unlikely intrinsic mechanisms alone can account for the long dormancy periods observed by DTCs in the bone marrow. Alternatively, the bone marrow microenvironment has been implicated as a supportive niche for the existence of disseminated breast cancer cells in a dormant state [19,20]. Breast cancer cells localized close to the endosteum, the interface of bone and marrow which serves as a supportive niche for hematopoietic stem cells and a frequent site of cancer cell dissemination, were shown to have long doubling times suggesting a possible quiescent state [21]. Intercellular communication through gap junctions between breast cancer cells and the bone marrow stroma close to the endosteum has recently been suggested to play a role in the maintenance of a dormant state [22]. Furthermore, *in vitro* studies have demonstrated an inhibitory effect on proliferation and acquisition of an invasive mesenchymal phenotype of breast cancer cells upon co-culture with bone marrow stroma isolated from breast cancer patients [15]. These findings illustrate the significance of the cellular interactions within the bone marrow microenvironment and the subsequent effects on the phenotype of disseminated breast cancer cells.

Recent reports have presented data supporting the bi-directional flow of DTCs, demonstrating targeted homing of DTCs to tumors present in the mammary fat pad and accelerated tumor progression upon colonization by the DTCs [23,24]. The early detection and persistence of DTCs in the bone marrow of breast cancer patients signifies the bone marrow microenvironment may function as a reservoir for DTCs [11]. It is highly probable that cancer cells within the bone marrow microenvironment will re-enter the circulation, disseminating to other organs or back to the primary site of tumor formation. Therefore DTCs in the bone marrow not only pose a threat to the development of metastatic lesions in the bone, but may also contribute to the development of metastases at other sites as well as tumor progression and/or recurrence at the primary site.

Although implicated in recurrence at the primary site of tumor formation and the development of metastatic disease, the malignant potential of dormant breast cancer cells residing in the bone marrow remains undetermined. We previously reported detection of early disseminated human breast cancer cells by measuring human DNA in the bone marrow of mice that harbored mammary fat pad tumors derived from injection with primary tumorspheres isolated from patient core biopsies. These early disseminated breast cancer cells were detected prior to the development of metastatic lesions that were detected by H+E staining for up to 12 months post-injections [25]. These findings prompted further investigation into the tumorigenicity and metastatic potential of DTCs within the bone marrow. Herein, we demonstrate a malignant and aggressive metastatic phenotype of dormant breast cancer cells isolated from the bone marrow of mice. These data offer compelling evidence that supports the crucial role of the bone marrow microenvironment in both the maintenance of dormancy and the conversion of breast cancer cells to a more aggressive and rapid growth phenotype once cells have exited the bone microenvironment.

## Materials and Methods

### Cell Culture

Tumorspheres were isolated using a procedure previously described by this laboratory [26] and derived from Dontu et al. [27]. Briefly, breast cancer needle biopsies from primary tumors were obtained during the routine care of patients with consent and Tulane IRB approved protocol (IRB # 07-00042). Tissues were mechanically and enzymatically dissociated then sequentially filtered through a 100  $\mu$ m and 40  $\mu$ m pore filter (Fisher). After washes with 1XPBS, the cell pellet was resuspended in DMEM/F12 media containing 1 $\times$  B-27 serum-free supplement (Invitrogen), 0.4% bovine serum albumin (BSA) (Sigma), 20 ng/ml epidermal growth factor (EGF) (Sigma), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma), 4  $\mu$ g/ml insulin, human recombinant (Sigma), and penicillin (100 U/ml)/streptomycin (100 U/ml) and cultured in a 100 mm<sup>2</sup> ultra low attachment plate (Corning). Cells were cultured for 10–14 days to allow tumorsphere formation. Cells were pelleted every 3 days by centrifugation at 300 $\times$ g for 10 min. and resuspended in complete DMEM/F12 media supplemented with fresh EGF and bFGF.

### Animal experiments

Immunodeficient Nu/Nu female mice were purchased from Charles River Laboratories (US). Mice were 25–35 days of age at time of injection. All experiments were performed under approved Tulane IACUC protocol (IACUC # 2941 R-D). Cold 1XPBS and a 27 gauge syringe was used to flush bone marrow from the femurs of mice previously injected with tumorspheres isolated from samples 5–9 into the mammary fat pad. Bone marrow was flushed from non-injected age-matched mice and injected into the mammary fat pad as a negative control. The flushed bone marrow was washed twice in cold 1XPBS. To perform a cell count, an aliquot of the isolated bone marrow was combined in a 1:1 ratio with 0.4% trypan blue stain (BioWhittaker) and loaded onto a hemocytometer. Immediately before injection, the bone marrow was combined with 100  $\mu$ l BD Matrigel Basement Membrane matrix (BD Biosciences). Mice were anesthetized by i.p. injection of 0.3 ml of a ketamine solution. Cell suspensions were injected bilaterally into the third mammary fat pad. Mice were monitored weekly for tumor formation by caliper measurement and for body weight for up to twelve months. If no weight loss or other indications of declining health were observed, animals were euthanized twelve months post injection.

### Hematoxylin and Eosin (H+E) Staining

Tissues were collected and placed in 10% neutral buffered formalin (Fisher) equal to 20 times the tissue volume. Tissues were incubated overnight at room temperature and then processed by standard formalin fixation, paraffin embedding and sectioning by The Center for Gene Therapy Histology Core Facility, Tulane University Health Sciences Center. 5  $\mu$ m sections were deparaffinized and rehydrated in a graded series of ethanol solutions, from 100% to 75%. Sections were then stained using Gill's Hematoxylin and Eosin (Poly Scientific) followed by dehydration through a graded series of ethanol solutions from 75% to 100%. Image J software was used to quantify the metastatic burden within the tissues analyzed. To calculate the metastatic burden present in the mouse organs, the number of pixels within the defined area of the metastatic lesion/s was determined (x pixels). Next, the total number of pixels within the field of view was determined (y pixels). The metastatic burden within the field of view was then calculated by dividing the pixels present in the metastatic lesion/s by the total pixels comprising the field of view

then multiplying by 100  $[(x \text{ pixels}/y \text{ pixels}) \times 100]$  resulting in a percent metastatic burden. The average of five fields of view (100 $\times$  magnification) was used to determine metastatic burden present in each organ analyzed.

### Immunohistochemistry (IHC) of tumors and mouse tissue

For IHC, tissues were collected and placed in 10% neutral buffered formalin equal to 20 times the tissue volume (Fisher). Tissues were incubated overnight at room temperature and then processed by standard formalin fixation, paraffin embedding and sectioning by The Center for Gene Therapy Histology Core Facility. IHC was performed using the Vectastain staining kit (anti-rabbit, PK6101; anti-mouse PK6102, Vector Laboratories). Briefly, 5  $\mu$ m sections were rehydrated (as described above) followed by heat-induced, epitope retrieval performed in a pressure cooker for 25 min. in Tris Buffer, pH 9 (Biocare Medical). To inactivate endogenous peroxidase, slides were incubated in 0.3% hydrogen peroxide followed by a 10 minute wash in dH<sub>2</sub>O then washed 3 $\times$ 3 min. each in PBS. Sections were incubated in blocking buffer (10% normal goat serum diluted in PBS) for 30 min. at room temp and subsequently incubated overnight at 4°C with primary antibody diluted in blocking buffer. Primary monoclonal antibodies used were E-cadherin (24E10, Cell Signaling), estrogen receptor  $\alpha$  (SP1, Thermo Scientific), and anti-human Human Nuclear Antigen (HNA) (MAB1281, Chemicon). Primary polyclonal antibodies used were  $\beta$ -catenin (9563, Cell Signaling) and fibronectin (ab2413, Abcam). The HNA antibody was human specific. Antibodies for E-cadherin, estrogen receptor  $\alpha$ ,  $\beta$ -catenin and fibronectin were cross-reactive with human and mouse proteins. The following day, sections were washed 2 $\times$ 5 min. in PBS-T. Biotinylated secondary antibody was added to the sections for an incubation period of 30 min, followed by 2 $\times$ 5 min. washes in PBS-T. Streptavidin/biotin HRP-conjugate was added to the sections for an incubation period of 30 min. at room temperature followed by 2 $\times$ 5 min. washes in PBS-T. The signal was developed for a time that did not exceed 2 minutes using the Vector DAB substrate kit, according to the manufacturers' instructions. Sections were dehydrated (as described above) and mounted using Permount (Fisher). Staining was visualized using a bright field microscope and IP lab software.

## Results

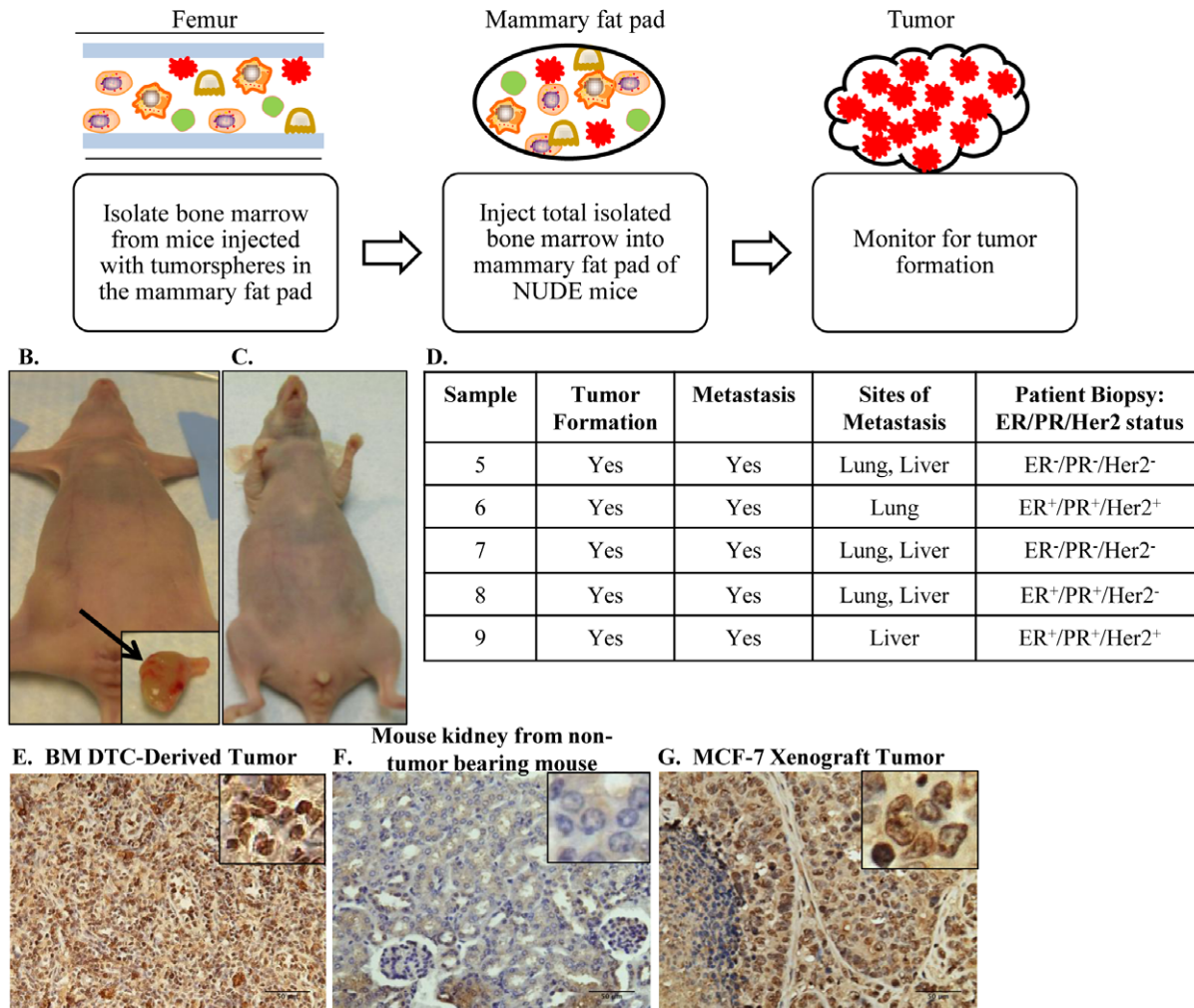
### Tumorigenicity of disseminated cancer cells in the bone marrow of mice previously injected with tumorspheres into the mammary fat pad

Our previous study used tumorspheres derived directly from breast cancer patient needle biopsies to establish primary tumors in the mammary fat pad of nude mice [28]. Two months post-injection of tumorspheres, human cancer cells were detected in the bone marrow of mice without the development of detectable metastatic lesions by H+E staining up to 12 months post-injection [25], suggesting the disseminated breast cancer cells persisted in a dormant state in the bone marrow microenvironment. However, the viability and tumorigenicity of the disseminated breast cancer cells detected in the bone marrow remained undetermined. Therefore, the purpose of this study was to determine the malignant and metastatic potential of the disseminated cancer cells within the bone marrow upon injection into the mammary fat pad.

Tumorspheres isolated from patient biopsy samples 5–9 formed small, palpable tumors upon injection of  $\leq 5 \times 10^3$  cells into the mammary fat pad. These tumors metastasized to the bone (femur), as detected by PCR for human-specific chromosome 17, however

did not result in the development of macrometastatic lesions up to 12 months post-injection [25]. Total bone marrow was flushed from the femurs of mice that were injected with tumorspheres isolated from patient core biopsy sample 5–9 into the mammary fat pad 8–10 months prior.  $12.5 \times 10^6$  total bone marrow cells (containing normal mouse bone marrow cells and human metastatic breast cancer cells) were injected into the mammary fat pad of NUDE mice (Figure 1A). Injection of  $12.5 \times 10^6$  total bone marrow cells from these femurs into the mammary fat pads of mice [referred to as sample 5, 6, 7, 8 or 9 BM (Bone Marrow)] resulted in the formation of large tumors in the mammary fat pad 2 months post-injection (Figure 1B, D). Injection of  $12.5 \times 10^6$  total bone marrow cells isolated from non-tumor bearing mice did not result in tumor formation in the mammary fat pad (Figure 1C). To determine whether normal mouse bone marrow cells affected tumor formation by tumorspheres,  $12.5 \times 10^6$  bone marrow cells from non-tumor bearing mice were co-injected with sample 5–9 tumorspheres that were previously shown to form small, palpable tumors [25]. Co-injection of normal mouse bone marrow cells with sample 5–9 tumorspheres did not affect primary tumor size (data not shown). Positive staining for HNA on 5  $\mu$ m paraffin-embedded sections of BM-derived tumors (representative micrograph of positive HNA staining of sample 5 BM tumor, Figure 1E) demonstrated that the majority of cells within the tumors were of human origin. Control sections of 5  $\mu$ m paraffin-embedded mouse kidney from a non-tumor bearing mouse did not exhibit positive HNA staining (Figure 1F), whereas 5  $\mu$ m paraffin-embedded sections of a human MCF-7 xenograft tumor exhibited positive HNA staining (Figure 1G) demonstrating the human specificity of the HNA antibody. In addition to being highly tumorigenic, samples 5–9 BM also exhibited metastatic potential with metastatic lesions detected in the lung and liver for sample 5, 7, and 8 BM, metastatic lesions detected in the lung only for sample 6 BM, and metastatic lesions detected in the liver only for sample 9 BM (Figure 1D). These data demonstrate that dormant metastatic breast cancer cells in the bone marrow were highly tumorigenic upon transplantation into the mammary fat pad, forming tumors that were significantly larger than tumors formed by injection of tumorspheres isolated from the original patient biopsies.

The tumors formed from the DTCs present in the injected bone marrow consisted of small tumor cells with pleomorphic nuclei that did not exhibit tubule formation (Figure 2A, B, C, D, E). Using IHC, various markers were evaluated to demonstrate the relative epithelial and mesenchymal features of the tumors. The cell adherens junction protein E-cadherin is normally expressed in the membrane of differentiated epithelial cells and more differentiated breast cancer cells.  $\beta$ -catenin, a central mediator of the WNT signaling pathway, binds to E-cadherin at the membrane in conjunction with a complex of proteins connecting the adherens junction to components of the cytoskeleton [29,30]. In the absence of membrane E-cadherin,  $\beta$ -catenin is either rapidly degraded or can translocate to the nucleus upon activation of WNT signaling. A low level of E-cadherin expression was variably detected in the nucleus in sample 5, 6 and 9 BM tumors (Figure 2F, G, and J inset, arrow), and no E-cadherin expression was detected in sample 7 and 8 BM tumors (Figure 2H and I).  $\beta$ -catenin expression was variably detected in the membrane and nucleus of sample 5, 6, 8 and 9 BM tumors (Figure 2K, L, N, O inset, arrow), with no expression detected in sample 7 BM tumors (Figure 2M). Fibronectin expression was detected in sample 5–9 BM (Figure 2P, Q, R, S, T). Sample 5–9 BM were negative for estrogen receptor alpha (ER $\alpha$ ) (data not shown). These data demonstrate that the tumors derived from dormant metastatic breast cancer cells in the bone marrow exhibited a mesenchymal-

**A. Disseminated breast cancer cells in the bone marrow are highly tumorigenic and metastatic**

**Figure 1. Tumor formation in the mammary fat pad upon injection of total bone marrow aspirates isolated from femurs containing metastatic tumor cells.** **A.** Experimental design to determine the tumorigenicity of disseminated human cancer cells in the bone marrow of mice previously injected with tumorspheres into the mammary fat pad. Bone marrow, aspirated from femurs of mice previously injected with tumorspheres into the mammary fat pad, was injected into the mammary fat pad of NUDE mice to determine the tumor-forming ability of dormant cancer cells in the bone marrow. **B.** Injection of  $12.5 \times 10^6$  cells/pad aspirated from the femurs of mice injected with tumorspheres resulted in large tumor formation in the mammary fat pad two months post-injection. **C.** Injection of  $12.5 \times 10^6$  cells/pad aspirated from the femurs of non-injected mice resulted in no tumor formation in the mammary fat pad three months post injection. **D.** Summary of tumor formation and metastasis for sample 5–9 BM, including ER/PR/Her2 status of patients samples from which the parental tumorspheres were first derived. **E.** Representative positive HNA staining of 5  $\mu$ m paraffin-embedded sections of sample 5 BM tumors demonstrated the presence of human cells. **F.** No positive nuclear HNA staining of 5  $\mu$ m paraffin-embedded sections of mouse kidney from non-tumor bearing mouse (negative control). **G.** Positive HNA staining of 5  $\mu$ m paraffin-embedded sections of a human MCF-7 xenograft (positive control). 200 $\times$  magnification in all panels.  
doi:10.1371/journal.pone.0047587.g001

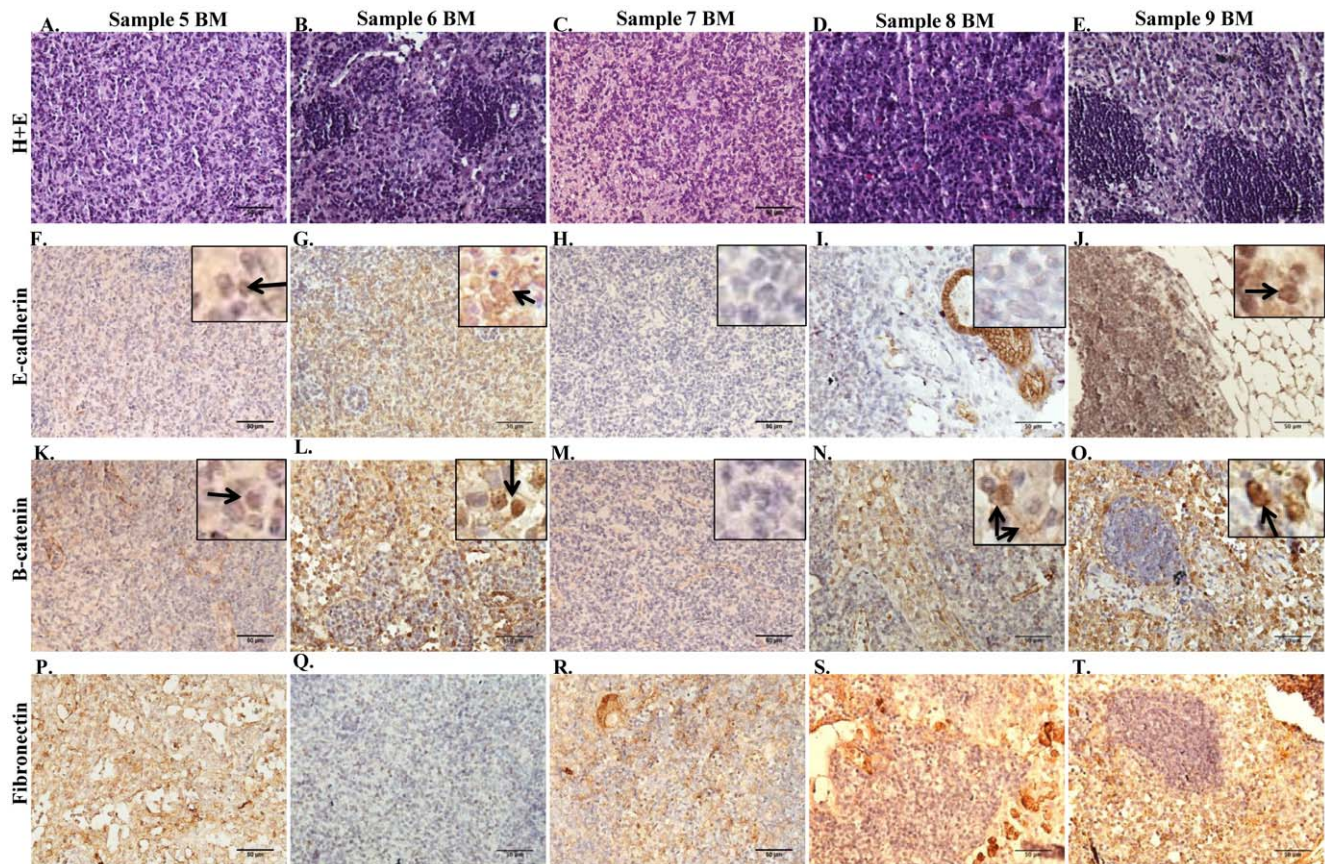
like phenotype when transplanted into the mammary fat pad of NUDE mice.

### Metastatic potential of disseminated cancer cells in the bone marrow upon injection into the mammary fat pad

Paraffin-embedded sections of lungs, kidneys, and livers were prepared from animals bearing primary tumors from sample 5–9 BM to determine the metastatic potential of the cells. Animals were euthanized for the collection of organs on the basis of tumor burden present in the mammary fat pad and/or declining health. Metastatic lesions were detected by H+E in the lung for sample 5–8 BM (Figure 3A, B, C, D, respectively), in the liver for sample 5, 7–9 BM (Figure 4A, B, C, D, respectively), and in the kidney for

sample 5–9 BM (data not shown). Nuclear E-cadherin was detected in the metastatic lesions of the lungs of sample 5, 7 and 8 BM (Figure 3E, G, H, respectively), however E-cadherin was detected predominantly in the membrane in metastatic lesions in the lung of sample 6 BM (Figure 3F). Metastatic lesions in the lung for sample 5 and 6 BM exhibited variable expression of  $\beta$ -catenin in the membrane and in the nucleus (Figure 3I and J, respectively), whereas metastatic lesions in the lung for sample 7 and 8 BM demonstrated variable nuclear expression only of  $\beta$ -catenin (Figure 3K and L, respectively). E-cadherin was detected in the membrane of metastatic cells in the liver of sample 5 and 8 BM (4E and G, respectively); in contrast no E-cadherin expression was detected in liver metastatic lesions of sample 9 BM (Figure 4H).





**Figure 2. Expression of markers for epithelial and mesenchymal lineages in tumor samples. A–E.** H+E staining of tumors formed in the mammary fat pad upon injection of bone marrow aspirated from the femurs of mice injected with tumorspheres isolated from samples 5–9 BM, respectively. **F–T.** Representative IHC demonstrating patterns of expression of E-cadherin (**F–J**),  $\beta$ -catenin (**K–O**), and fibronectin (**P–T**) in sample 5–9 BM tumors. 200 $\times$  magnification in all panels.  
doi:10.1371/journal.pone.0047587.g002

The majority of metastatic cells in the liver for sample 7 BM demonstrated nuclear E-cadherin expression (Figure 4F), however E-cadherin was detected in the membrane of a small population of metastatic cells as well (data not shown). In conjunction with the detection of E-cadherin in the membrane, metastatic cells in the liver for sample 5 and 8 BM exhibited variable  $\beta$ -catenin expression in the membrane (Figure 4I and K, respectively). However,  $\beta$ -catenin expression was not detected in the metastatic cells in the liver for sample 7 and 9 BM (Figure 4J and L, respectively). Fibronectin expression was detected in metastatic lesions in the lung for sample 5–7, and 9 BM and metastatic lesions in the liver for sample 5, 7–9 BM (data not shown). These data indicate that the metastatic cells within the liver for sample 5 and 8 BM exhibited an epithelial-like phenotype, however metastatic cells for sample 5, 7 and 8 BM in the lung and sample 7 and 9 BM in the liver maintained a mesenchymal-like phenotype similarly to that observed within the primary tumors in the mammary fat pad.

#### Organ tropism of the metastatic cells and the metastatic burden within the mouse organs

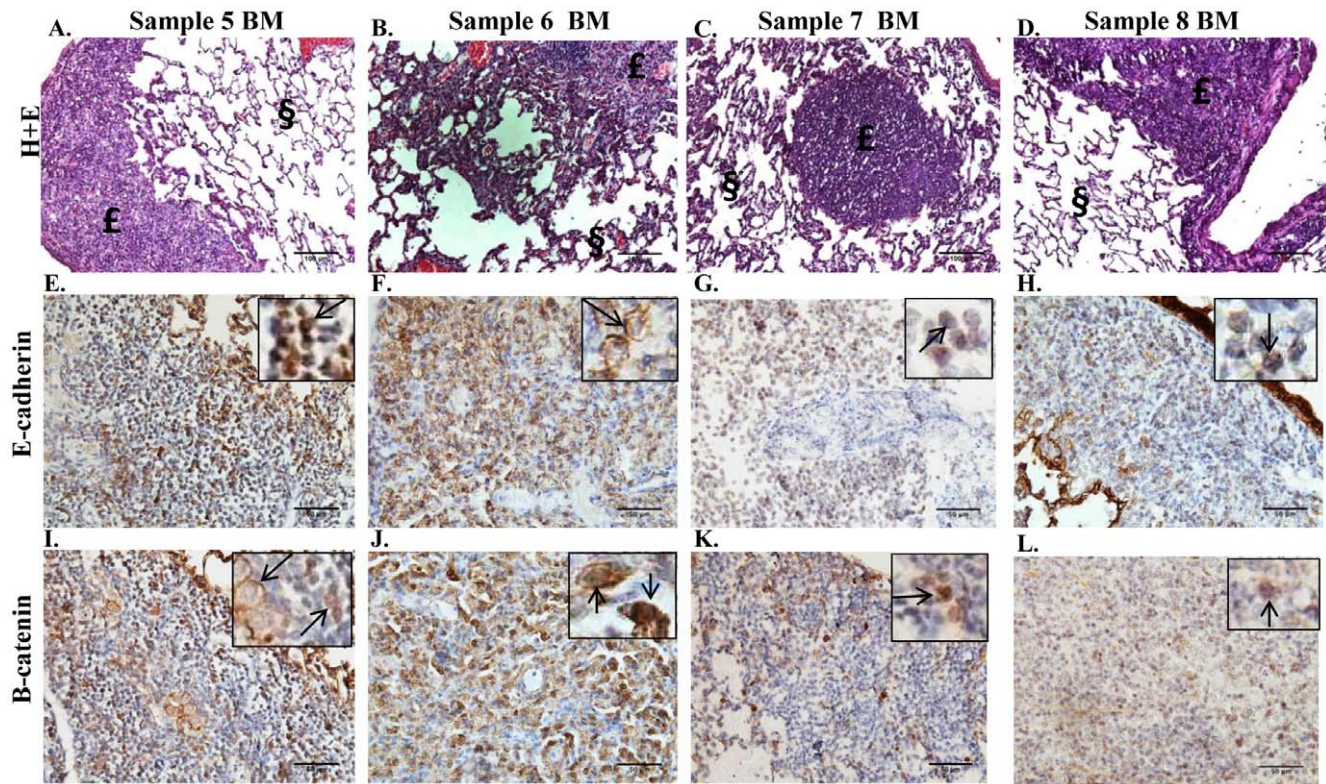
A comprehensive analysis of metastasis was performed at the time of necropsy (upon excessive tumor burden and/or moribund condition) to compare tropism of each sample to different organs and quantify the relative metastatic burden within each organ for each sample as a measure of the ability of metastatic cells to

colonize organ sites with outgrowth into larger lesions. It is likely that metastatic lesions were present prior to termination of the experiments. **Future experiments will remove organs at various time intervals post-injection to determine the time to development of metastatic lesions in various organs.** To determine differences in tissue-specific tropism between samples, the number of each organ with detectable metastases by H+E staining (lung, kidney and liver) was counted without regard to size of the metastatic lesion. The metastatic burden within each organ was then quantified as described in the material and methods. The number (n) of lungs, kidneys and livers analyzed for each sample is indicated in Figure 5.

The tissue-specific tropism (without regard to the size of the metastatic lesions) was comparable between sample 5–9 BM (data not shown). Sample 5–9 BM exhibited greater metastatic burden within the lung and liver as compared to the kidney, with the overall largest metastatic burden detected in the lung (Figure 5A). In comparison to their parental cell population (tumorspheres isolated from patient core biopsies) for which the average metastatic burden measured for sample 5–9 was below 20%, the DTC-derived metastatic lesions in the lung and the liver exhibited a higher metastatic burden with an average metastatic burden of 35% in the lung and 25% in the liver. The majority of metastatic lesions were detected between 50–150 days post-injection of bone marrow into the mammary fat pad for sample 5–9 BM (Figure 5B). In comparison to the metastatic phenotype demonstrated by



## Lung Metastasis



**Figure 3. Metastatic lesions in the lungs of mice bearing mammary fat pad tumors that were derived from transplantation of bone marrow aspirate containing metastatic tumor cells.** A–D. H+E staining performed on 5  $\mu$ m paraffin-embedded sections of lung from sample 5–8 BM illustrates metastatic lesions. Metastatic lesions indicated by £; normal mouse tissue indicated by §. 100 $\times$  magnification. E–H. IHC performed on 5  $\mu$ m paraffin-embedded sections of lung from sample 5–8 BM using a monoclonal antibody to E-cadherin. I–L. IHC performed on 5  $\mu$ m paraffin-embedded sections of lung from sample 5–8 BM using a polyclonal antibody to  $\beta$ -catenin.  
doi:10.1371/journal.pone.0047587.g003

sample 5–9 tumors derived from tumorspheres isolated from patient biopsies [25], sample 5–9 BM had a similar overall metastatic phenotype however exhibited an accelerated development of large metastatic lesions.

## Discussion

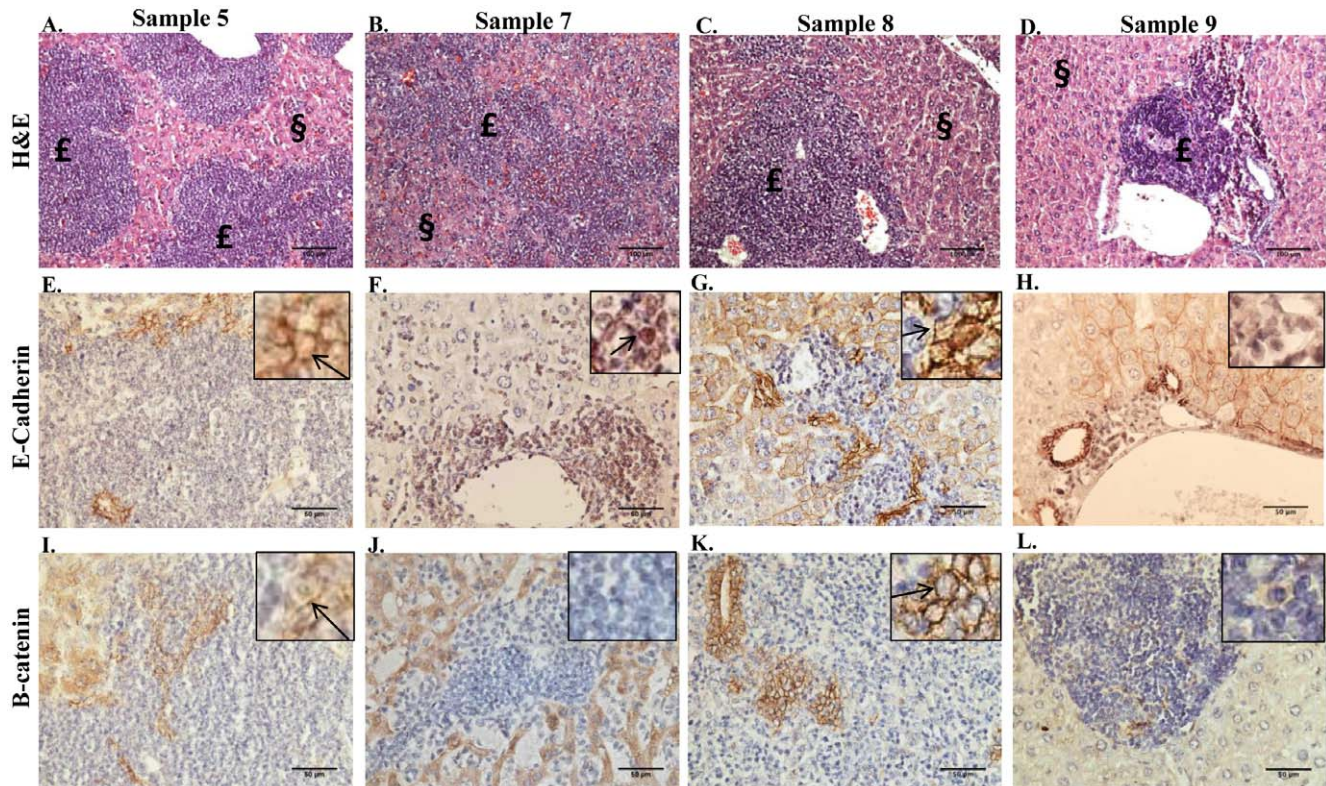
The bone microenvironment has long been considered to play an important role in the dormancy of disseminated breast cancer cells [11,15,31,32]. However, low numbers of disseminated cells and inaccessibility has hindered studies aimed at elucidating the cellular and molecular mechanisms contributing to dormancy of cancer cells residing in the bone marrow microenvironment. Presented in this study is the first evidence of the malignant potential of dormant breast cancer cells in the bone marrow that had metastasized from a primary tumor in the mammary fat pad derived from primary bTICs. The absence or low expression of epithelial markers, including E-cadherin and  $\beta$ -catenin, and the elevated expression of fibronectin within the tumors indicated that the cells adopted a more mesenchymal phenotype. However, changes in the expression patterns of E-cadherin and  $\beta$ -catenin within metastatic lesions present in the lung and liver suggested that the cells retained a level of plasticity, enabling adaptation and survival at the distant sites of metastasis. The injection of dormant disseminated breast cancer cells present in the bone marrow resulted in the formation of larger primary tumors in the mammary fat pad, and accelerated development (up to 300 days

earlier) of large metastatic lesions within the lung, liver and kidney as compared to their “parental” tumorsphere cell population that were derived directly from patient biopsies [25]. Taken together, these data demonstrate that dormant human breast cancer cells residing in the bone marrow microenvironment exhibit a highly malignant and aggressive metastatic phenotype when removed from the bone and transplanted into the mammary fat pad of NUDE mice.

In our previous study, we demonstrated the formation of small tumors in the mammary fat pad upon the injection of tumorspheres isolated directly from patient core biopsies [25]. The tumors became palpable 3 months post-injection and maintained a volume of about 100 mm<sup>3</sup> for up to 12 months post-injection. From these small tumors, cells disseminated to the bone (femur) and entered a state of dormancy for up to 12 months post-injection, with a small number of cells detected by PCR for human chromosome 17, but without the development of larger metastatic lesions that could be detected by H+E staining. Taking into consideration the malignant capacity of the “parental” population of cells within the tumorspheres, and the dormant state of cells within the bone marrow, it is remarkable that injection of bone marrow containing a small population of disseminated cancer cells into the mammary fat pad resulted in the formation of large tumors within 2 months post-injection. The aggressive malignant phenotype exhibited by dormant tumor cells in bone marrow highlights the importance of the biological changes occurring



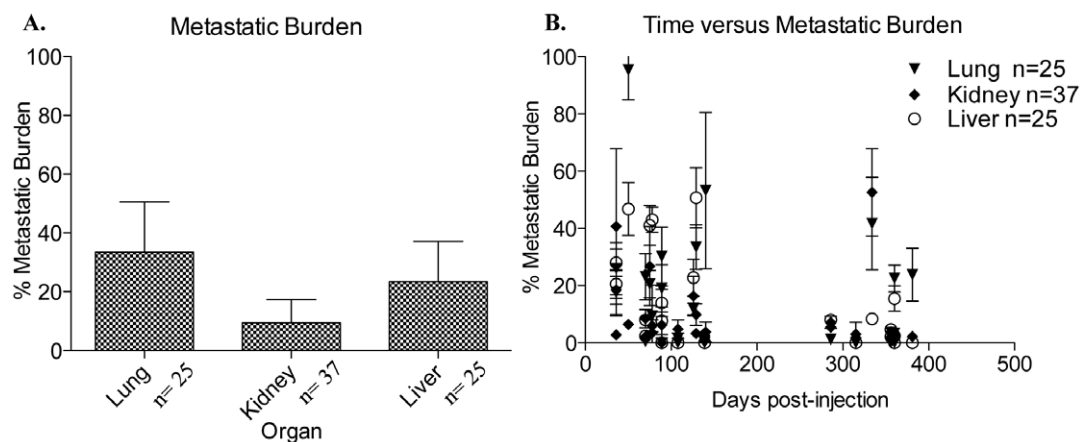
## Liver Metastasis



**Figure 4. Metastatic lesions in the livers of mice bearing mammary fat pad tumors that were derived from transplantation of bone marrow aspirate containing metastatic tumor cells.** A–D. H+E staining performed on 5 µm paraffin-embedded sections of liver from sample 5–7, and 9 BM illustrates metastatic lesions. Lesion indicated by £; normal tissue indicated by §. 100× magnification. E–H. IHC performed on 5 µm paraffin-embedded sections of liver from sample 5–7, and 9 BM using a monoclonal antibody to E-cadherin. I–L. IHC performed on 5 µm paraffin-embedded sections of liver from sample 5–7, and 9 BM using a polyclonal antibody to  $\beta$ -catenin. doi:10.1371/journal.pone.0047587.g004

during metastatic latency, indicating that dormancy is not static but rather the cells are continuously responding and adapting to

signals within the microenvironment. Comparing the gene expression profiles of the tumorsphere-derived tumors and BM



**Figure 5. Metastatic profile of bone marrow transplantation experiments.** A. Graphical representation of the metastatic burden determined for the lungs, kidneys, and livers analyzed by H+E staining from samples 5–9 BM. The metastatic burden in each organ was calculated by dividing the pixels present in the metastatic lesion/s (x pixels) by the total pixels comprising the field of view (y pixels) then multiplying by 100 [(x pixels/y pixels)\*100] resulting in a percent value. Values are reported as mean  $\pm$  SD. B. Graphical representation of the percent metastatic burden, previously calculated as above, compared to days post-injection of total bone marrow into the mammary fat pad for sample 5–9 BM. Values are reported as mean  $\pm$  SD. doi:10.1371/journal.pone.0047587.g005

DTC-derived tumors could reveal the molecular mechanisms contributing to the aggressive phenotype of DTCs post-metastatic latency in the bone marrow. Furthermore, these data indicated a vital role of stromal and/or cellular components within the bone marrow microenvironment for the persistence of disseminated breast cancer cells in a dormant state during metastatic latency.

To determine whether the non-tumorigenic mouse bone marrow was conferring a growth advantage for tumor formation, tumorspheres isolated from sample 5–9 were co-injected into the mammary fat pad with  $12.5 \times 10^6$  bone marrow cells isolated from non-injected mice. The co-injection with non-tumorigenic mouse bone marrow did not confer a growth advantage for tumor formation by the tumorspheres; the resulting tumors were similar in size to the small tumors formed by injection of tumorspheres alone. Therefore, the presence of resident mouse bone marrow cells did not account for the malignant phenotype exhibited by the small population of cancer cells present in the isolated bone marrow. However, since the cellular and molecular interactions within the bone marrow microenvironment likely have reciprocal effects on the resident bone marrow cells and the cancer cells, the bone marrow from a non-injected mouse does not control for possible changes in the bone marrow cells.

Previous studies have shown that the majority of DTCs in bone reside within the endosteal niche and vascular niche [33–35]. The endosteal and vascular niches are dynamic specialized compartments with cellular and stromal compartments that contribute to the maintenance and differentiation of hematopoietic stem cells (HSCs) [36–38]. HSCs are in close association with osteoblasts in the endosteum that together provide signals to maintain HSCs in a primitive, quiescent state and provide an anchor to the endosteal niche [39–41]. Migration into the vascular niche from the endosteal niche stimulates the regulated proliferation, differentiation and mobilization of HSCs/HPCs (hematopoietic progenitor cells) to the peripheral circulation [42,43]. The molecular interactions between the cellular and stromal compartments in the maintenance of HSC quiescence may similarly contribute to the dormancy of cancer cells residing within the endosteal niche. It has been hypothesized that breast cancer dormancy in bone is due to interactions with resident cells within the bone marrow microenvironment, such as mesenchymal stem cells, stromal cells, and osteoblasts (reviewed in [44]). Reactivation of disseminated breast cancer cells for recurrence may occur due to changes in cell-cell signaling, increasing genetic instability, and/or migration of the cancer cells to a different niche within the bone marrow. However, the mechanisms for breast cancer dormancy in bone and tumor recurrence remain unknown. The inherent requirement for the components of the endosteal niche and vascular niche to be responsive to exogenous changes in the environment renders these niches as possible targets for therapeutic manipulation [37]. Investigation into the reciprocal biological changes within the endosteal niche and the cancer cells may elucidate molecular targets within the microenvironment for the eradication of DTCs prior to the development of macroscopic lesions.

The hormone receptor status of the DTCs in the bone marrow is often altered when compared to the original primary tumor. Fehm *et al.* reported 71% of patients with estrogen receptor alpha ( $ER\alpha^+$ ) primary tumors had  $ER\alpha^-$  DTCs in the BM [45] and Dietsch *et al.* reported only 2 out of 11 patients with  $ER\alpha^+$  primary tumors had  $ER\alpha^+$  DTCs in the BM [46]. In contrast, patients with  $ER\alpha^-$  primary tumors presented predominantly with  $ER\alpha^-$  DTCs in the BM [45]. Isolation of tumorspheres from patient samples under non-adherent, serum-free conditions enriches for breast stem/progenitor cells, or breast-tumor-initiating cell (bTICs). It is hypothesized that recurrent and metastatic

disease are predominantly derived from the less differentiated cells with stem-like characteristics that would retain the ability to produce progeny with changes in the expression of key markers, resulting in the observed alterations in  $ER\alpha$  expression in recurrent and metastatic disease as compared to the primary tumor. Interestingly, both the tumorsphere-derived tumors and their metastases and the DTC-derived tumors and their metastases were negative for  $ER\alpha$  despite the expression of  $ER\alpha$  in patient tumor samples 6, 8 and 9. **Future experiments will determine the expression of  $ER\alpha$  within the tumorspheres isolated from the patient samples, as well as the expression of  $ER\alpha$ , EMT markers and stem cells markers within early disseminated tumor cells within the bone marrow and other sites of metastasis. Insight into the phenotype of early disseminated tumor cells in the bone marrow could lead to the identification of novel targets for the eradication of DTCs during dormancy prior to the development of recurrent and metastatic disease.**

The loss of E-cadherin and  $\beta$ -catenin expression in the membrane of epithelial cells can be indicative of an epithelial to mesenchymal transition (EMT) [47,48]. It is hypothesized that the metastatic process is initiated when tumor cells undergo either a full or partial EMT, leading to the acquisition of mesenchymal characteristics such as invasiveness, anchorage-independent growth, and resistance to apoptosis [49,50]. The aberrant and/or low levels of expression of E-cadherin and  $\beta$ -catenin in sample 5–9 BM tumors suggested that the cells with malignant potential acquired some features of a mesenchymal phenotype. If dormant tumor cells residing in the bone marrow microenvironment exhibited a more mesenchymal phenotype, this could have important implications on current techniques employed to detect DTCs in the bone marrow of patients, which predominantly detect epithelial markers such as cytokeratins [7,17], Her2/neu [51], Epcam [52], and Mucin [6]. **The identification of new markers for the detection of biologically relevant DTCs with tumorigenic potential may improve the prognostic value of DTCs in the bone marrow of breast cancer patients in terms of local and distant recurrence.**

Metastasis to the lung, liver and kidney demonstrated that the dormant tumor cells within the bone marrow that formed primary tumors in the mammary fat pad retained a similar metastatic profile as the “parental” cells within the tumorspheres; however the expression patterns of E-cadherin and  $\beta$ -catenin within the metastatic lesions in the lung and liver derived from DTC-derived tumors differed from the lesions in the lung and liver derived from tumorsphere-derived tumors [25]. Nuclear localization of E-cadherin has been predominantly observed in pituitary adenomas [53], esophageal squamous cell carcinoma [54], Merkel cell carcinoma [55], solid pseudopapillary tumor of the pancreas [56,57], clear-cell renal cell carcinoma [58], colorectal cancer (and its liver metastases) [54] and ovarian granulosa cell tumors [59]. Although nuclear localization of E-cadherin has not conclusively been described in breast cancer, one study identified nuclear E-cadherin expression in 21% of FNAC smears from breast carcinomas [60]. Nuclear expression of E-cadherin was found to strongly correlate with higher grade tumors with more aggressive biological behavior in the same study [60]. The down-regulation of E-cadherin in breast tumors is usually a consequence of transcriptional regulation or promoter methylation and is associated with invasion, metastasis and an overall worse prognosis for patients [61–64]. However the extracellular and cytoplasmic domain of E-cadherin has been shown to undergo proteolytic cleavage, suggesting another regulatory mechanism of E-cadherin in tumors. Cleavage of the extracellular domain of E-cadherin results in the release of a soluble 80 kDa fragment that has been



shown to disrupt cell-cell junctions by antagonizing the full length E-cadherin [65,66]. In addition, the extracellular fragment of E-cadherin has been shown to bind to and activate Her2 and Her3 cell signaling [67]. Interestingly, cleavage products of E-cadherin were detected by Western blot in protein extracts prepared from DTC-derived tumor samples (Figure S1). **Cleavage of E-cadherin at the membrane resulted in an 80 kDa extracellular fragment and 37 kDa intracellular fragment supporting the observation of nuclear localization of E-cadherin by IHC.**

MMP-3 and MMP-7 are two proteases implicated in the extracellular cleavage of E-cadherin. Interestingly, the secretion of active MMP-3 and MMP-7 by tumorspheres isolated from samples 5–9 was detected by zymography (unpublished results) however the role of MMP-3 and/or MMP-7 in the aberrant expression of E-cadherin observed in this model has yet to be determined. The cytoplasmic domain of E-cadherin can undergo proteolytic cleavage by caspase-3 and calpain, translocate to the nucleus and influence cell signaling [65,68–70]. However, the mechanisms by which E-cadherin translocates to the nucleus and its potential role in the regulation of gene expression remain unknown. The aberrant expression of E-cadherin detected within the tumorsphere-derived tumors [25], as well as within the DTC-derived tumors and their metastatic lesions in the lung and liver (as presented in this study), **provides initial evidence of nuclear E-cadherin in primary breast cancer cells associated with metastasis and other cellular processes. The nuclear localization of E-cadherin warrants further investigation to determine its possible role in tumor progression, metastasis and dormancy. Future studies will be aimed at determining the mechanism by which the cytoplasmic fragment of E-cadherin enters the nucleus, possible interactions with other proteins in the nucleus and any effects on transcription.**

The disseminated cancer cells within the bone marrow metastasized to the lung, liver and kidney, as well as the brain and spleen (data not shown). Although the metastatic profiles of the present BM DTC experiments and tumorsphere experiments from our previous study [25] were comparable, there were a few noted differences. The most notable difference between the BM DTC experiments and the tumorsphere experiments [25] was the accelerated development of detectable macrometastatic lesions in the lung, liver and kidney from the BM DTCs. Whereas the majority of metastatic lesions for sample 5–9 were detected at  $\geq 200$  days post-injection of tumorspheres into the mammary fat pad [25], the majority of metastatic lesions for sample 5–9 BM were detected at  $\leq 150$  days post-injection. The larger primary tumor size and the significantly earlier development of metastases for the BM DTC-derived tumors suggests that the disseminated breast cancer cells in the bone marrow acquired a proliferative advantage through residence in the bone marrow as compared to cells in the tumorsphere-derived tumors. These data may suggest that cells within the bone marrow have the potential to further disseminate to other organs with the proliferative capacity to aggressively form metastatic lesions. Alternatively, the resident bone marrow cells may undergo pre-conditioning as a result of crosstalk with the DTCs or alterations in the microenvironment caused by the presence of the DTCs in the bone. An eloquent study by Kaplan *et al.* demonstrated the recruitment of VEGFR1<sup>+</sup> bone marrow derived cells (BMDCs) to tumor-specific sites of metastasis, resulting in the establishment of a pre-metastatic niche for the colonization of disseminated tumor cells [71]. This previous study demonstrated the crucial role of BMDCs in the early steps of metastasis. Therefore, changes within the cells in the bone marrow during metastatic latency may contribute to the highly aggressive metastatic phenotype observed by DTCs in the bone marrow upon injection of total bone marrow into the mammary fat pad.

**Investigation into the differences between disseminated tumor cells from tumorsphere-derived tumors and BM DTC-derived tumors, as well as changes within the resident bone marrow cells during metastatic latency, may elucidate important pathways involved in cancer cell dormancy.**

The organ microenvironment has been implicated in this study as well as previous studies as a possible target in the treatment of metastatic disease. There would be two predominant desired outcomes upon the manipulation of the organ microenvironment: maintenance of dormancy or induced exit from dormancy. On the one hand, the organ microenvironment could be manipulated by exogenous signals to prevent the exit of DTCs from dormancy in an attempt to prevent the development of macroscopic lesions. Alternatively, suppression of the mechanisms contributing to dormancy or activating the signals permitting the exit from dormancy would eliminate metastatic latency allowing the use of cytostatic/cytotoxic therapies to target the proliferating metastatic tumor cells. The results presented in this study suggest that DTCs acquire a highly aggressive and malignant phenotype during metastatic latency indicating that it may be dangerous to permit the persistence of DTCs in a dormant state for extended periods of time. Although cytotoxic and/or cytostatic therapies have been shown to be ineffective in treating metastatic disease, the lack of suitable models has hindered investigation into the efficacy of either approach. **Using the model presented in the present study, future research can investigate the treatment of metastatic disease in a systems biology approach, providing a means to determine potential effects on DTCs through manipulation of the organ microenvironment.**

Disseminated breast cancer cells in the bone are known to exist in a dormant state for extended periods of time, maintaining the ability to proliferate upon activation to form overt clinical lesions [44,72,73]. **However the mechanisms contributing to the maintenance of dormancy and subsequent exit from dormancy have yet to be determined.** Although the presence of DTCs in the bone marrow provides a strong prognostic indicator for breast cancer, many patients remain relapse-free even after 10 years. In this study, we have presented data implicating the vital role of extrinsic factors within the bone microenvironment in the dormancy of disseminated cancer cells. We demonstrate the malignant potential and aggressive metastatic profile of dormant cancer cells in the bone marrow, suggesting stable modifications of DTCs occur within the bone marrow microenvironment that facilitate malignancy once cells exit the bone. **Future studies investigating the reciprocal cross-talk between disseminated cancer cells and resident cells within the bone marrow microenvironment will begin to expose the mechanisms involved in the regulation of cancer cell dormancy that may lead to improved detection and eradication of DTCs in the bone.**

## Supporting Information

**Figure S1 Western blot analysis of tumors demonstrating extracellular and intracellular cleavage products of E-cadherin.** Western blot analysis of protein isolated from sample 5 BM tumors demonstrates the presence of the 80 kDa extracellular cleavage product and 37 kDa cytoplasmic cleavage product of E-cadherin. (TIF)

## Author Contributions

Conceived and designed the experiments: CGM BGR. Performed the experiments: CGM LC. Analyzed the data: CGM KM BGR. Contributed reagents/materials/analysis tools: MJW BGR. Wrote the paper: CGM BGR.



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# Chapter 23

## Breast Tumor-Initiating Cells Isolated from Patient Core Biopsies for Study of Hormone Action

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### Abstract

In recent years, evidence has emerged supporting the hypothesis that cancer is a stem cell disease. The cancer stem cell field was led by the discovery of leukemia stem cells (Tan, B.T., Park, C.Y., Ailles, L.E., and Weissman, I.L. (2006) The cancer stem cell hypothesis: a work in progress. *Laboratory Investigation*. **86**, 1203–1207), and within the past few years cancer stem cells have been isolated from a number of solid tumor including those of breast and brain cancer among others (Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 3983–3988; Singh, S.K., Clarke, I.D., Terasaki, M., Bonn, V.E., Hawkins, C., Squire, J., and Dirks, P.B. (2003) Identification of a Cancer Stem Cell in Human Brain Tumors. *Cancer Research*. **63**, 5821–5828). Cancer stem cells exhibit far different properties than established cells lines such as relative quiescence, multidrug resistance, and multipotency (Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H.M., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006) Cancer Stem Cells-Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Research*. **66**, 9339–9344). In addition, our laboratory has demonstrated that breast cancer stem cells exhibit a strong metastatic phenotype when passaged in mice. Since stem cells exhibit these somewhat unique properties, it will be important for endocrinologists to evaluate hormonal action in these precursor cells for a more thorough understanding of cancer biology and development of more effective treatment modalities. A relatively easy and low cost method was developed to isolate breast cancer stem cells from primary needle biopsies taken from patients diagnosed with primary invasive ductal carcinoma during the routine care of patients with consent and IRB approval. Fresh needle biopsies (2–3 biopsies at 2 cm in length) were enzymatically dissociated in a collagenase (300 U/ml)/hyaluronidase (100 U/ml) solution followed by sequential filtration. Single cell suspensions were cultured on ultra low attachment plastic flasks in defined medium and formed non-adherent tumorspheres. The tumorspheres exhibited surface marker expression of CD44<sup>+</sup>/CD24<sup>low/-</sup>/ESA<sup>+</sup>, previously defined as a “breast cancer stem cell” phenotype by Al Hajj et al. (Al-Hajj M., Wicha M.S., Benito-Hernandez A., Morrison, S.J., and Clarke, M.F. (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 3983–3988).

**Key words:** Breast cancer, tumor-initiating cells, primary cell culture, tumorspheres, invasive ductal carcinoma, cancer stem cells.

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## 1. Introduction

An understanding of the mechanisms for hormone action and endocrine therapy response in breast cancer is limited due to the complexity of this disease, the numerous disease subtypes, and *de novo* and acquired resistance to endocrine therapies. Part of the complexity in breast cancer is likely due to the presence of limiting numbers of cancer stem cells. By definition, cancer stem cells are tumor cells that (a) have the ability to self-renew and (b) can recapitulate the entire cellular heterogeneity of tumor from which the cells were derived when transplanted into an immunodeficient mouse model (1). In this regard, the cancer stem cell would be the progenitor of all the differentiated tumor cells that constitute the bulk of a breast tumor. Whereas the more differentiated breast tumor cells are sensitive to chemotherapy and endocrine therapy for breast cancer, the cancer stem cells are insensitive to these therapeutics by virtue of a relatively quiescent phenotype, expression of membrane pumps, and uncertain nuclear receptor status.

Tumor initiating cells (T-ICs), a term used to describe a putative stem cell population, are a population of cells that contain a sub-population of cancer stem cells (1). T-ICs have the capacity to initiate and maintain tumor growth; however the term refers to a more heterogeneous population of cells containing a population of cancer stem cells (1). Isolation of T-ICs will provide an important laboratory tool to understand endocrine regulation of these multipotent cells. This chapter describes methods for isolation, culturing, and characterization of breast T-ICs.

Breast T-ICs can be isolated using a variety of methods. Fluorescence activated cell sorting (FACS) utilizes the expression of identified cell surface markers to isolate T-ICs (2,3). The advantage of using FACS to isolate breast T-ICs is that the initial cell population will be less heterogeneous. However, propagation *in vitro* as tumorspheres will most likely yield a heterogeneous population of cells ranging from cancer stem cells to more differentiated progenitor cells. The low number of cells recovered from FACS is a caveat of the technique, depending on the size of the tissue and hence the total number of cells initially isolated from the primary tissue. For the isolation of breast T-ICs, an alternative to isolating T-ICs from primary tissue is FACS sorting of established breast cancer cell lines (4). This method offers accessibility and convenience due to the unlimited resource of cell lines. However, similar to the parental cell line, T-ICs sorted from cancer cell lines present translational limitations and complications from long-term culture.

The method presented in this chapter for isolating breast T-ICs is more accessible for researchers without consummate stem cell experience and cheaper than alternative methods described above.

The following method is a variation of a valid isolation method developed by Ponti et al. (5) and uses specific culture conditions to isolate non-adherent cells that form tumorspheres in vitro. One disadvantage to the following method is that a heterogeneous population of cells is isolated as compared to FACS sorting to isolate population based on specific cell surface markers. However this method provides a robust and reliable approach to isolate T-ICs for studies in which the degree of “stemness” of isolated cells is not as critical as the tumor formation efficiency and the serial transplantability. Isolated primary T-ICs provide a tumor model that more closely recapitulates the primary tumor when injected into the mammary fat pad of immunodeficient mice. For that reason, tumor models derived from T-ICs are more relevant for investigation of basic tumor biology, metastasis, and response to hormones and therapeutics.

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## 2. Materials

### ***2.1. Isolation of Breast T-ICs from Biopsy Core Samples***

1. 1x Hanks' balanced salt solution (HBSS) with phenol red.
2. 1x Phosphate buffered solution (PBS), pH 7.0.
3. Dulbecco's Phosphate Buffered Saline (DPBS), sterile-filtered, cell culture tested (Sigma-Aldrich).
4. 7.5% bovine serum albumin (BSA) in DPBS.
5. Disposable scalpels.
6. Dulbecco's Modified Eagles Medium/Ham's F-12 (DMEM: F-12): 1:1 mix, with L-glutamine and 15 mM HEPES (#11330-032, Invitrogen).
7. 10x Collagenase (3000 U/ml)/Hyaluronidase (1000 U/ml) in Dulbecco's Modified Eagle's Medium with glucose (1000 mg D-Glucose/L) (Stem Cell Technologies).
8. Sterile Petri dishes.
9. BD Falcon Filter Cell Strainer, 100  $\mu$ m and 40  $\mu$ m pore size.
10. B-27 supplement (50X) (GIBCO-Invitrogen).
11. Human recombinant epidermal growth factor (EGF) is dissolved at 20  $\mu$ g/ml in 10 mM acetic acid/0.1% BSA and stored in 500  $\mu$ l aliquots at  $-20^{\circ}\text{C}$ . The working concentration is 20 ng/ml and therefore used at a 1:1000 dilution.
12. Insulin, human recombinant (SAFC Biosciences) diluted in autoclaved ddH<sub>2</sub>O at 400  $\mu$ g/ml, using hydrochloric acid to adjust the pH to dissolve (about pH 2.7). Aliquots of 400  $\mu$ g/ml stored at  $-20^{\circ}\text{C}$  and used at a working concentration of 4  $\mu$ g/ml.

13. Human recombinant basic fibroblast growth factor (bFGF) is dissolved at 25 µg/ml in DMEM:F-12 and stored in aliquots at -20°C. The working concentration is 10 ng/ml.
14. DMEM:F-12 complete medium. To prepare 500 ml of DMEM:F-12 complete medium, add 5 ml of 400 µg/ml of human recombinant insulin, 500 µl of 20 µg/ml human EGF, 200 µl of 25 µg/ml of bFGF, 26.7 ml of 7.5% BSA in DPBS, and 10 ml of 50X B-27 supplement to 447.6 ml of DMEM:F12. Sterile filter the final solution (*see Note 1*).
15. Ultra low attachment culture 75 cm<sup>2</sup> flasks/100 mm dishes/6-well plates/24-well plates/96-well plates (Corning), depending on the number of cells and types of experiments to be performed.

## **2.2. Culture Conditions and Maintenance In Vitro**

1. DMEM:F-12 as in 2.1.
2. B-27 supplement (50X).
3. hEGF (20 ng/ml).
4. Insulin (4 µg/ml).
5. Human bFGF (10 ng/ml).
6. 7.5% BSA in DPBS.
7. Ultra low attachment culture 75 cm<sup>2</sup> flasks/100 mm dishes/6-well plates/24-well plates/96-well plates (Corning), depending on the number of cells and types of experiments to be performed.
8. 1x Trypsin with EDTA (0.05%) (Invitrogen).
9. 1x Trypsin inhibitor from *glycine max* (soybean) stored in 1–2 ml aliquots at -20°C (Sigma-Aldrich).

## **2.3. Immunocytochemistry on Isolated Tumorspheres**

1. 1x PBS with 2% fetal bovine serum (FBS), pH 7.4.
2. 1x PBS with 1% BSA, pH 7.4.
3. Cytocentrifuge (Cytospin 2, StatSpin).
4. Filter concentrators (FF01/FF01-B) and stainless steel clips (FFCL, StatSpin).
5. Cardboard filters (supplied with concentrator and used to concentrate the cells at one spot on the slide).
6. Superfrost plus microscope slides, pre-cleaned.
7. Acetone.

## **2.4. Immunocytochemistry on Tumorsphere Cytospins**

1. 1x PBS, pH 7.4.
2. Normal goat serum.
3. Nonidet-P40 (NP40), reagent grade.

4. Humidified chamber; an unused pipette tip box with wet paper towels and a small amount of ddH<sub>2</sub>O in the bottom.
5. IgG1-FITC/IgG2a-PE antibody mixture (Beckman Coulter). This mixture of two nonspecific antibodies that are isotype matched for the CD44-PE antibody and CD24-FITC antibodies is used as a negative control for nonspecific staining. If using different primary antibodies then choose the appropriate isotyped matched IgG control antibody to assess nonspecific staining.
6. CD44-PE antibody and CD24-FITC antibody (BD Biosciences).
7. ESA-FITC antibody (Biomeda).
8. Hoechst 33342 dye (1 mM) to be diluted in 1x PBS, pH 7.4 to a working concentration of 10  $\mu$ M.
9. Prolong Gold Antifade reagent.
10. Coverglass slips 24  $\times$  30 mm.

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### 3. Methods

Isolating primary breast T-ICs from patient core biopsies can be challenging. With an approved IRB protocol and patient consent, tumor biopsies may be obtained either at the time of initial patient diagnosis or following tumor resection. During initial patient diagnosis, ultrasound guided needle biopsies are efficient for obtaining core biopsies from the tumor and not the surrounding normal tissue. However all resultant cultures must be evaluated by a pathologist for cellular and nuclear morphology to determine whether cells are from cancer or normal tissue origin. There is always variability in the number of T-ICs isolated from different biopsies. Regardless of the variability, a limited number of T-ICs will be isolated from a small amount of tissue such as a core biopsy. Alternately, biopsies obtained by the surgeon following tumor resection (True-cut biopsies) may also be used as a source for T-ICs and would provide a larger amount of starting material. Once again, resection samples would have to be evaluated by a pathologist to ensure separation of cancerous tissue from normal tissue prior to isolating T-ICs. A further concern for resection samples would occur if the patient received any neoadjuvant therapy prior to resection that could introduce variability in the T-ICs. It has been demonstrated that neoadjuvant chemotherapy may actually enrich for the number of T-ICs obtained from resection

biopsies (6). Nonetheless, cultures established from resection samples should be carefully evaluated with the expectation that not all tissue samples will yield T-ICs.

*Due to the possible infectious nature of the patient tissue samples, safety precautions are needed during dissociation of the tissue and during all subsequent culturing and handling of the samples. The user should consult with the appropriate safety officials for proper protection against infectious agents.* Since there is no penicillin/streptomycin added to the complete medium, precise and careful cell culture techniques need to be employed. In addition, if there are established cell lines present in the same cell culture laboratory, primary cells should be used in a separate hood and designated incubator that will not contain established cell lines to minimize cross contamination.

### **3.1. Isolation of Breast T-ICs from Biopsy Core Samples**

1. Upon receipt of core biopsies, immediately place the biopsy pieces into a 15 ml conical tube containing 1x HBSS (4°C) and place on ice for immediate transport to the laboratory.
2. Once back in the laboratory, transfer the core biopsy pieces and 1x HBSS into a sterile Petri dish in a sterile tissue culture hood.
3. Thaw 3–5 ml (depending on the amount of tissue) of 10x collagenase/hyaluronidase in a 37°C water bath. Pre-warm DMEM:F-12 in a 37°C water bath for diluting the 10x collagenase/hyaluronidase.
4. Remove the 1x HBSS from the dish and discard into a 50 ml conical tube containing bleach (*see Note 2*).
5. Wash the core biopsies twice with 1x PBS, pH 7.4. Removing and disposing of the 1x PBS should be done in the same manner as the 1x HBSS in 3.1.4.
6. Place the core biopsies into a new sterile Petri dish (or a sterile tissue culture plate) and add 8–10 ml of 1x PBS, pH 7.4. Using two sterile disposable scalpels mince the tumor pieces into ~2 mm pieces in 1x PBS, pH 7.4. Do not use excessive mechanical dissociation of the tissue or increased cell death will occur.
7. Transfer the minced tissue pieces in PBS to a 50 ml conical tube. Allow the tumor pieces to settle to the bottom (about 5–10 minutes).
8. While waiting, dilute the pre-thawed 10x collagenase/hyaluronidase to 1x in pre-warmed DMEM:F-12 and mix thoroughly.
9. From step 3.1.7, remove the 1x PBS supernatant without disturbing the settled minced tumor pieces and dispose the PBS in bleach as in step 3.1.4.
10. Add 30–50 ml of 1x collagenase/hyaluronidase to the minced tumor pieces and gently agitate, either by pipetting up and down or by brief and slow pulse on a vortexer (*see Note 3*).



11. Incubate the 50 ml conical tube containing the tumor pieces in a 37°C water bath for 3–4 hours, agitating every 15–25 minutes by pipetting up and down in the tissue culture hood. If an overnight incubation for the enzymatic digestion is preferred, additional growth factors etc. must be added to the DMEM:F-12/collagenase/hyaluronidase mixture to prevent excessive cell death. Usually, 3–4 hour enzymatic dissociation at 37°C is sufficient for enzymatic dissociation.
12. Prepare DMEM:F-12 complete medium during the enzymatic dissociation step (*see* 2.1.13).
13. Upon completion of the enzymatic dissociation, sequentially filter the cell suspension through a 100 µm pore filter, and then a 40 µm pore filter and collect the flow through into a fresh 50 ml conical tube. This step is used to remove undigested tissue and clumps of cells that will not pass through the filters and should be discarded. The flow through will contain single cells that will be used in the next step.
14. Centrifuge the resultant single cell suspension at 350g for 10 minutes. Resuspend the cell pellet in complete medium with gentle pipetting. Remove 10 µl of the resuspended cell solution for cell counting by trypan blue exclusion.
15. Plate 1000 cells/ml in an ultra low attachment plate/flask (6), depending on the total number of isolated cells in the single cell suspension. Culture cells at 37°C with 5% CO<sub>2</sub>. Tumorspheres should be visualized by light microscopy within 3–6 days of culture. A general flow chart of the procedure and an image of a tumorsphere are shown in **Fig. 23.1**. Maintain tumorspheres under non-adherent culture conditions in the DMEM:F-12 complete media.

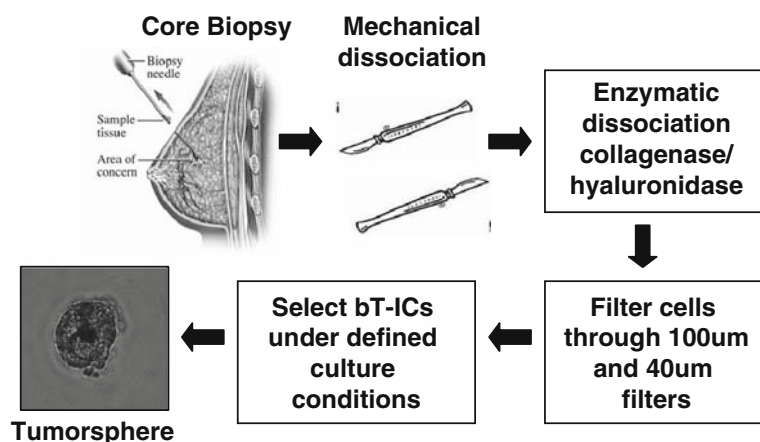


Fig. 23.1. A flow chart illustrating the general isolation procedure for bT-ICs including an image of a “tumorsphere” in vitro. bT-ICs; breast tumor initiating cells.

### **3.2. Culture Conditions and Maintenance In Vitro**

1. When approximately 50% of the tumorspheres reach 60  $\mu\text{m}$  in diameter, collect all tumorspheres in a 50 ml conical tube and centrifuge at 300*g* for 10 minutes (tumorspheres that are larger than 60  $\mu\text{m}$  in diameter develop a darkened center).
2. While cells are in the centrifuge, thaw a sufficient number of aliquots of trypsin inhibitor in a 37°C water bath.
3. Carefully aspirate the supernatant (*see Note 4*) and gently resuspend the cells in 1 ml of 0.05% trypsin-EDTA keeping bubble formation to a minimum.
4. Incubate the 50 ml conical tube containing the cells in a 37°C water bath for 10 minutes with gentle agitation after 5 minutes.
5. After the 10 minute incubation, add an equal volume of trypsin inhibitor to the cells (e.g., if cells were incubated in 1 ml of trypsin, add 1 ml of trypsin inhibitor). Pipette the cells up and down about 40 times using a Rainin P1000 pipettor with sterile plugged pipette tips, minimizing bubble formation.
6. Centrifuge the cells at 350*g* for 10 minutes (*see Note 5*).
7. Gently resuspend the cell pellet in complete media with a Rainin P1000 pipettor and sterile plugged pipette tips (keeping bubble formation to a minimum). Remove 10  $\mu\text{l}$  of the resuspended cell solution for cell counting by trypan blue exclusion.
8. Plate the cells at 1000 cells/ml on ultra low attachment plates/flasks in DMEM:F-12 complete medium.
9. Every 3 days, collect cells by centrifugation at 350*g* for 10 minutes and replat cells with fresh complete media containing EGF and bFGF.

### **3.3. Tumorsphere Cytospins**

1. Harvest >100 tumorspheres in a conical tube and centrifuge at 300*g* for 10 minutes.
2. Wash (pipette up and down 3–4 times) the tumorspheres in cold 1x PBS, pH7.4 supplemented with 2% FBS twice, centrifuging at 300*g* for 10 minutes after each wash.
3. After the second wash, resuspend the tumorspheres in 100  $\mu\text{l}$  of 1x PBS, pH7.4 supplemented with 1% BSA (using cotton plugged sterile pipette tips) and keep tumorspheres on ice.
4. Label the Superfrost Plus microscope slides appropriately. Be aware of the slide orientation when placing slides into the cytocentrifuge to avoid loading cells into the incorrect wells of the filter concentrators. Once assembled, it is very difficult to see the labels on the slides.
5. Assemble the filter concentrators and cardboard filters as per the manufacture instructions. Cardboard filters are used to concentrate the cells at one spot on the glass slide. Be sure that

the hole in the cardboard filter is in the proper position and the filter and the slide are flush to ensure the cells can reach the slide.

6. Place the assembled filter concentrators into the appropriate positions in the cytocentrifuge.
7. Add 100  $\mu$ l of 1x PBS, pH7.4 supplemented with 1% BSA to the wells of the filter concentrators (*see Note 6*). Centrifuge at 500*g* for 2 minutes.
8. Quickly add 100  $\mu$ l of each tumorsphere sample to the appropriate wells of the filter concentrators. Centrifuge at 500*g* for 10 minutes.
9. Remove the filter concentrator apparatus from the cytocentrifuge. Remove the metal clips being careful not to scrape the filter across the slide with the attached cells (the circle of liquid on the slide indicates the location of the cells on the slide).
10. Carefully open the filter concentrator separating the cardboard filter from the slide without any lateral motion that would disturb the attached cells on the slide.
11. Dry the slides overnight at room temperature (in a secure location to avoid any disturbance).
12. Add acetone to the slides for 5 minutes at 4°C to fix the cells.
13. Wash the slides three times with 1x PBS, pH 7.4 for 5 minutes each wash at room temperature.
14. Dry slides at room temperature but avoid excessive drying (*see Note 7*). Store slides at -20°C if staining won't be performed immediately.

### **3.4.** ***Immunocytochemistry*** ***on Tumorsphere*** ***Cytospins***

1. Blocking step: using the slides from step 3.3.14, add ~200  $\mu$ l of blocking buffer (10% normal goat serum and 0.2% NP-40 in 1x PBS, pH 7.4) to slides. Incubate slides for 30 minutes in a humidified chamber at room temperature.
2. During the 30 minute blocking step, prepare primary antibody dilutions in blocking buffer (*see step 3.4.1*). For antibody incubations, at least 200  $\mu$ l of diluted antibody will be needed per slide. Antibody dilutions should be determined empirically. As an example, CD44-PE, CD24-FITC, and ESA-FITC antibodies were diluted 1:100 in blocking buffer. All experiments should make use of appropriate controls (incubation of slides with isotype matched nonspecific antibody or peptide competition of primary antibody). As an example, a mixture of IgG1-FITC and IgG2a-PE (Beckman Coulter) served as isotype matched control antibodies for CD44-PE, CD24-FITC, and ESA-FITC. Control antibodies should be prepared at the same dilution in blocking buffer

(or the same mass/volume) as the primary antibodies. Some primary antibodies may be incubated concomitantly (e.g., CD44-PE and CD24-FITC antibodies may be incubated together). Keep all antibodies on ice and protected from light (*see Note 8*).

3. Wash the slides twice with cold 1x PBS, pH 7.4, blotting the slides on paper towels to remove the excess 1x PBS, pH 7.4 after each wash. Be sure to only touch the edge of the slide to the paper towel to avoid disturbing the attached tumorspheres.
4. Turn off as many lights in the workspace as possible before working with fluorophore-conjugated antibodies. Prepare your workspace such that exposure to light during the addition of the diluted antibodies is minimized.
5. Add ~200  $\mu$ l of each antibody dilution to the appropriately labeled slides.
6. Cover the humidified chamber with aluminum foil without tipping the chamber, since this may cause loss of liquid from the desired location on the slide.
7. Incubate the slides in the humidified chamber for 90 minutes at room temperature.
8. Dilute 1 mM of Hoechst dye (*see Note 9*) 1:100 in 1x PBS, pH 7.4 to make a 10  $\mu$ M working solution. Make enough working solution to add 500  $\mu$ l to each slide. Keep working solution on ice and protect from light.
9. Remove the Prolong Gold Antifade reagent from  $-20^{\circ}\text{C}$  to equilibrate to room temperature before use.
10. Wash slides twice with 1x PBS, pH 7.4 for 5 minutes each. Blot the edge of the slides on a paper towel to remove excess 1x PBS, pH 7.4 after each wash.
11. Add ~500  $\mu$ l of 10  $\mu$ M Hoechst 33342 dye to each slide. Incubate at room temperature for 15–20 minutes, in the humidified chamber with aluminum foil.
12. Wash slides once with 1XPBS, pH 7.4.
13. Blot the side of the slides gently on a paper towel to remove excess 1x PBS, pH 7.4.
14. Using a Pasteur pipette, make a thin line of the Prolong gold antifade reagent along one edge of the slide, adjacent to the attached tumorspheres. Pick up a coverslip using a forceps and touch the edge of the coverslip to the thin line of the Prolong gold antifade reagent. Gently drop the coverslip onto the slide. Once the Prolong gold reagent has dispersed under the coverslip, check for bubbles. If there are excess bubbles, use the forceps to gently push the bubbles to the edge of the coverslip for removal.

15. Allow the preparation to cure overnight, protected from light, before imaging. Images of the staining, including controls, are shown in **Fig. 23.2**.

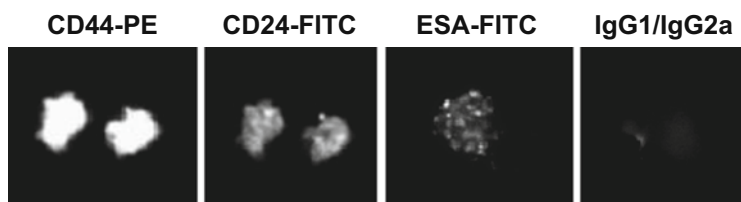


Fig. 23.2. Immunocytochemistry of stained cytopins of tumorspheres derived from breast cancer biopsy demonstrating a  $CD44^{+}/CD24^{low/-}/ESA^{+}$  phenotype.

#### 4. Notes

1. Replace the growth factors, EGF and bFGF, every 3–4 days. Store complete media for up to 1 month at 4°C, protected from light.
2. Before beginning, setup several 50 ml conical tubes containing bleach. All reagents and supplies that come into contact with the tissue pieces should be placed into the bleach before disposal.
3. For two core biopsies, typically 30 ml of 1x collagenase/hyaluronidase is sufficient for complete enzymatic digestion. For each additional core biopsy, in general, increase the amount of 1x collagenase/hyaluronidase by 10 ml.
4. Depending on the number and size (from 40 to 100  $\mu\text{m}$ ) of the tumorspheres in culture, a pellet may or may not be visible following centrifugation. Prior to centrifugation, mark the region of the tube where the pellet should form. If there is no pellet visible, aspirate the majority of the supernatant and then hold the tube at a 15–45° angle to the working surface of the tissue culture hood while pressing the aspirating pipette to the side of the tube to aspirate the supernatant at the bottom of the tube where the cells are pelleted (although not visible). Do not hold the tube at a sharp angle to aspirate for any length of time as the cells may detach from the bottom of the tube. In addition, do not repeatedly agitate the supernatant at the bottom of the tube while aspirating at an angle since this will detach cells from the bottom of the tube and the number of cells recovered will be significantly reduced.
5. Some protocols filter the dissociated cells through a 50–40  $\mu\text{m}$  pore filter at this step to ensure a single cell suspension. We do not include this step because of cell loss during filtration. For the purpose of tumorsphere formation assays, flow

cytometry and any other assays that require a single cell suspension with the exclusion of doublets, triplets, etc. should include this filtration step. However, for the sole purpose of passaging and expanding the tumorspheres in culture, filtration is not necessary at this step.

6. If a significant number of tumorspheres were obtained and cell loss is not a concern, wetting the filter with 1x PBS, pH7.4 supplemented with 1% BSA is not necessary. Wetting the filter with 1x PBS, pH7.4 supplemented with 1% BSA before adding the tumorspheres will increase the number of cells that are deposited on the slide following centrifugation.
7. Allow slides to dry just enough to prevent crystal formation from residual PBS during storage at  $-20^{\circ}\text{C}$ . Avoid excessive drying of the slide as this could decrease the efficiency and quality of subsequent staining.
8. The fluorophores conjugated to the various antibodies are photosensitive. For optimal visualization of the fluorescent signal, the antibodies should be protected from light throughout the procedure.
9. Hoechst 33342 is the preferred Hoechst dye used for this protocol. However other Hoechst dyes that are not cell permeable will also function well with this protocol since the cells were permeabilized during the fixation step with acetone and subsequent incubation with blocking buffer that contains the membrane solubilizing detergent NP-40.

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